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UGANDAN MEDICINAL PLANTS USED TRADITONALLY FOR ORAL CARE:
Investigation of Extracts for Anti-bacterial, Cytotoxic and Anti-inflammatory Effects

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Cover illustration: *Zanthoxylum chalybeum* plant species.
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UGANDAN MEDICINAL PLANTS USED TRADITONALLY FOR ORAL CARE:
Investigation of Extracts for Anti-bacterial, Cytotoxic and Anti-inflammatory Effects

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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When DISCOURAGEMENT comes, DON'T STOP; DIG DEEP and FIGHT it through! We fight through our FEARS and FAILURES by MAXIMIZING our FORWARD MOTION."

T .B. Joshua

Dedicated to my beloved family

ABSTRACT

The use of traditional medicine for treating human diseases remain widespread in low-resource settings. Medicinal plants form the backbone for traditional medicine systems in many parts of the world. In Uganda, most communities rely on medicinal plants to prevent and treat oral diseases. Despite the long history and widespread use of these plants in oral health care, there is limited scientific evidence on the efficacy and safety of these plants. The aim of this thesis was to investigate various extracts from the medicinal plants for anti-bacterial, cytotoxic and potential anti-inflammatory effects.

The thesis is based on three studies. Study 1 investigated 16 commonly used plant species from different parts of Uganda. Pulp juice from fresh plant materials and solvent extracts (hexane, methanol and water) from dry plant materials were obtained and tested for their anti-bacterial effects on periodontopathic bacteria *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia* and cariogenic bacteria *Streptococcus mutans*, *Streptococcus sobrinus, Lactobacillus acidophilus*. Study II assessed the essential oil extracts from ten Ugandan aromatic medicinal plants for growth inhibitory effects on periodontopathic bacteria *A. actinomycetemcomitans, P. gingivalis,* and cariogenic bacteria *S. mutans* and *L. acidophilus.* Chemical compositions of the oils were explored by gas chromatography–mass spectrometry. Study III investigated the cytotoxicity of essential oils from four promising plants (*Bidens pilosa, Ocimum gratissimum, Cymbopogon nardus, Zanthoxylum chalybeum*) that had shown strong anti-bacterial effects on Gram-negative periodontopathic bacteria *A. actinomycetemcomitans, P. gingivalis* in Study II. Potential effects of the oils on the production of pro-inflammatory cytokines (IL-6, IL-8 and prostaglandin E_2) by human gingival fibroblasts induced by IL-1β were also investigated.

In study I, the major findings were that fresh pulp juice prepared from *Zanthoxylum chalybeum* and *Euclea latidens* showed activity against all the bacterial species tested and at all strengths. Hexane extract from the aerial part of *Helichrysum odoratissimum* exhibited the best activity with MIC ranging from 0.0125 to 0.5mg/ml on all the bacterial species tested except *A. actinomycetemcomitans*. Methanol extract from *Lantana trifolia* was active on all the bacteria tested with MIC ranging 0.25 to 1mg/ml. In study II, the major findings were that essential oil from *Cymbopogon nardus* exhibited the highest activity with complete growth inhibition of *A. actinomycetemcomitans, P. gingivalis* at all the three concentrations tested, the major

constituents in the oil being mainly oxygenated sesquiterpenes. Essential oils extracted from *Teclea nobilis. Hoslundia opposita, Ocimum gratissimum* and *Bidens pilosa* were also considered promising because they showed marked growth inhibitory effects on least 2 bacterial species. In study III, essential oil from Z. *chalybeum* was the most cytotoxic while the oil from *C. nardus* the least cytotoxic. Essential oil from O. *gratissimum* significantly decreased baseline and induced secretion of Prostaglandin E_2.

We conclude that fresh extracts from the roots of *Z. chalybeum* and *E. latidens* had anti-bacterial effects on the cariogenic and periodontopathic bacteria, confirming the use of chewing sticks prepared from these plants as a tool for brushing teeth. The hexane extract from the aerial part of *H. odoratissimum* had strong anti-bacterial effects on cariogenic and periodontopathic bacteria, suggesting the potential use of this extract, or isolated active principles, in the treatment or prevention of dental caries and periodontal diseases. The marked inhibitory effects of the essential oils on periodontopathic bacteria *A. actinomycetemcomitans* and *P. gingivalis* suggest these oils could be avenues to explore for possible application in treatment of periodontal diseases. The essential oil extracted from *O. gratissimum* demonstrated both anti-bacterial and anti-inflammatory effects suggesting the oil could be worth exploring for possible application in treatment of periodontitis.

Key words: Medicinal plants, periodontal diseases, dental caries, essential oils, oral pathogens, human gingival fibroblast, cytotoxicity, pro-inflammatory cytokines.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following scientific papers which are referred to in the text by their roman numerals:

I. **Francis Ocheng**, Freddie Bwanga, Moses Joloba, Ann-Karin Borg-Karlson, Anders Gustafsson, Celestino Obua. Anti-bacterial activities of extracts from Ugandan medicinal plants used for oral care. Journal of Ethnopharmacology, 2014, 155, Pages 852–85.

II. **Francis Ocheng,** Freddie Bwanga, Moses Joloba, Abier Softrata, Muhammad Azeem, Katrin Pütsep Anna-Karin Borg-Karlson, Celestino Obua, Anders Gustafsson. Essential oils from Ugandan aromatic medicinal plants: Chemical composition and growth inhibitory effects on oral pathogen. Evidence-Based Complementary and Alternative Medicine, volume 2015, Article ID 230832, 10 pages.

III. **Francis Ocheng**, Freddie Bwanga, Elisabeth Almer Boström, Moses Joloba, Ann-Karin Borg-Karlson, Tülay Yucel-Lindberg, Celestino Obua, Anders Gustafsson. Essential oils from Ugandan medicinal plants: in *vitro* cytotoxicity and effects on IL-1-β induced pro-inflammatory cytokines and chemokine by human gingival fibroblasts (Manuscript).

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LIST OF ABBREVIATIONS

A	Asteraceae
ANOVA	Analysis Of Variance
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
B.pi	Biden pilosa
C.ci	Cymbopogon citratus
CCUG	Culture Collection, University of Göteborg
CFU	Colony Forming Units
C.na	Cymbopogon nardus
COX	Cyclooxygenase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
EUCAST	European Committee for Antimicrobial Susceptibility Testing
FBS	Fetal Bovine Serum
GC-MS	Gas Chromatography–Mass Spectrometry
HE	Hexane Extract
HPLC	High-Performance Liquid Chromatography
H.od	Helichrysum odoratissimum
H.op	Hoslundia opposita
IC_{50}	Half Maximal Inhibitory Concentration
IL-1β	Interleukin-1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
ISO	International Organization for Standardization

L	Lamiaceace
L.tr	Lantana trifolia
MBC	Minimum Bactericidal Concentration
MCP-3	Monocyte Chemotactic Protein-3
ME	Methanol Extract
MIC	Minimum Inhibitory Concentration
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
NCTC	National Collection of Type Cultures
N.D	Not Determined
NIST	National Institute of Standards and Technology
NO synthase	Nitric Oxide synthase
O.gr	Ocimum gratissimum
P	Poaceae
PGE_2	Prostaglandin E_2
PGES	Prostaglandin E synthase
R	Rutaceae
Tnf-α	Tumor necrosis factor-alpha
T.no	Teclea nobilis
V	Verbencaeace.
V.am	Vernonia amygdalina
WHO	World Health Organization
WE	Water Extract
Z.ch	Zanthoxylum chalybeum

1 INTRODUCTION

1.1 General overview

The use of traditional medicine for treating human diseases remain widespread in many parts of the world. World Health Organization (WHO) estimates that approximately 80% of the population in low-income countries rely mainly on traditional medicines for their primary healthcare (WHO, 2002a). In Uganda, it is estimated that approximately 60% of the population depend largely on traditional medicine for their health care needs (WHO, 2002a).

Traditional medicine is also referred to as "alternative medicine," "complementary medicine" or "ethno medicine" (WHO, 2008), and the use of plants (herbs) is a core part of all systems of traditional medicine (Kim, 2005). The WHO Congress on Traditional Medicine meeting in Beijing in 2008 acknowledged the importance of traditional medicine in the delivery of healthcare services especially in low-income regions like Sub-Saharan Africa, and further declared that systems of traditional medicine like plants should be further developed based on research and innovation (WHO, 2008). And indeed medicinal plants have become the focus of intense research, not only to find out whether their traditional uses are supported by actual pharmacological effects, but also because plants have proven to be sources of biologically active compounds, many of which have been the basis for the development of new lead chemicals for pharmaceuticals (Fabricant and Farnsworth, 2001; Wachtel-Galor and Benzie, 2011). The existence of a huge market for minimally processed medicinal plant materials (herbal preparations) especially in developed countries is also giving impetus to this research focus. The herbal preparations in many of these developed countries are commonly dispensed as over-the-counter medications and herbal supplements and are now very popular and of significance in primary health care (Wachtel-Galor and Benzie, 2011). Medicinal plants are used for treatment of a number of conditions, including oral diseases (Palombo, 2011).

Oral diseases continue to be a major public health problem worldwide, with dental caries and periodontal diseases being among the most common (Marcenes et al., 2013; Petersen et al., 2005). In spite of the general improvement in the overall health status of the population in

developed countries, including oral and dental health, the prevalence of dental caries in school aged children is reported to be up to 90%, with the majority of adults also affected (Petersen et al., 2005). In Uganda, a recent survey indicate dental caries prevalence of 33.5% in children and 66.7% in adults (Kutesa et al., 2015). A recent review also found that severe periodontitis affect 11% of the world population (Kassebaum et al., 2014). In Uganda, the severe form of periodontitis is reported to affect 6% of the Ugandan youth (Albandar et al., 2002).

Whereas most developed countries have tackled the burden of oral diseases through establishment of organized oral healthcare systems which offer curative and preventive services (Petersen et al., 2005), in low-income countries (including Uganda), however , access to curative oral healthcare services is limited because of low investments in oral health facilities. Where facilities are available, the high cost of treatment still make it out of reach to the majority of people (Ministry of Health, 2009; Muhirwe, 2006). The situation is further complicated by limited community-oriented prevention programs. The oral health practitioners needed to treat the most common oral conditions are also in short supply and the few available prefer to work in urban areas where the population that can afford treatment live. This deprive the rural population of even the most basic emergency treatment (Muhirwe, 2006). For a large number of the rural population in Uganda, medicinal plants offer the only available treatment option for dental and other related ailments. Most of the plants used are species indigenous to the region, and these may be gathered from the wild for self-medications, obtained from traditional healers or purchased from herb sellers.

The use of medicinal plants for treatment of diseases, including oral, have been practiced by many people in Uganda and is still being widely practiced. Information on how a particular plant is used and the disease it treats is normally passed on orally from generation to generation. This kind of information transmission is bound to distortion or loss whenever a medicine man dies without passing it to another person. To prevent such information distortion or loss, a survey was carried in Uganda with the main purpose of documenting and keeping permanent records of the different medicinal plants that are traditionally used in the treatment of various diseases, including oral and dental diseases (Mubiru et al., 1994). That study, however, stopped at the documentation and did not go further to test whether the traditional uses of these plants are supported by their actual pharmacological effects. There have also been several other reports on the use of medicinal plants in the traditional treatment of oral diseases in Uganda

(Hamill et al., 2003; Hirt and M'Pia, 2008; Kokwaro, 1993; Odongo et al., 2011), but all with limited or no laboratory studies to prove pharmacological effects. This thesis therefore emanated as a result of an effort to fill this knowledge gap. With the acceptance of medicinal plants as an alternative form of oral health care (Anonymous, 2000; WHO, 2002b) and renewed interests in natural products, there was an urgent need to evaluate the efficacy of these plants if they are to be utilized to their full potentials.

The main focus of the thesis was the efficacy of the plants in treatment periodontal diseases and dental caries. Aspects of cytotoxicity and-inflammatory effects were also investigated for promising plants extracts.

We envisaged that demonstration of anti-bacterial effects of fresh plant extracts (pulp juice) from some of the plants against bacteria associated with dental caries and periodontal diseases would promote the use of these plants as chewing sticks by the community (Study I). Demonstration of anti-bacterial effects of solvent and essential oil extracts could acts as leads for further development of these extracts into modern dental care products (Study I and II). Further, it is now well known that much of the tissue destruction that occur in periodontal diseases is as a result of the inflammatory reaction in the gingival tissues which is initiated by bacteria or their products. But for long, treatment of periodontal diseases has focused on elimination of bacterial infection, with little or no attention given to the inflammatory reaction. It is therefore not surprising that most patients do not respond well to such treatment approach, as they continue to experience ongoing inflammation, tissue damage, and eventual tooth loss. Because of this, current research is now focusing on discovering drugs/compounds that are endowed with both anti-bacterial and anti-inflammatory activities for the treatment of periodontal diseases (Sreenivasan and Gaffar, 2008). It was against this background that the thesis also looked at the anti-inflammatory effects of the extracts with promising activities especially on the bacteria implicated in the initiation of periodontal diseases (Study III). Demonstration of extracts with both anti-bacterial and anti-inflammatory attributes would be a basis for further study and development of these extracts into perio-care products. On the other hand, we argued that extracts from some of these plants could be having unfavorable effects on the gingival tissues and hence that was the basis for investigating the cytotoxicity of the promising extracts (Study III). Efforts were also made to identity the bioactive molecules in some of the extracts (Study II).

1.2 Effect of extracts from medicinal plants on oral pathogens.

Multiple studies from different parts of the world suggest that medicinal plants contain substances that promote dental health. Some of these studies have focused on oral bacteria implicated in the etiology of dental caries and periodontal diseases. In the Middle East, where the use of chewing sticks or miswak made from the desert plant *Salvadora persica* (Family: Salvadoraceae) is widespread, in *vitro* studies have shown that aqueous extract prepared from *S. persica* exerts anti-bacterial effects on some cariogenic oral bacteria, such as *Streptococcus mutans, Streptococcus fecalis*, *Streptococcus salivarius* and *Lactobacillus* sp. (Al-sieni, 2014; Almas, 1999). It has also been shown that *S. persica* has strong anti-bacterial effects on Gram-negative oral bacteria *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* which are fundamental in the initiation and progression of periodontal diseases (Sofrata et al., 2008). Clinical studies comparing the mechanical effect of *S. persica* miswak and modern tooth brushing on plaque removal reported that miswak is as effective as tooth brushing in reducing plaque and gingivitis (Al-Otaibi et al., 2003). In addition to its mechanical effect, *S. persica* miswak exerts chemical activity against bacteria and plaque formation (Al-Otaibi et al., 2004). Studies have also shown that extracts from *S. persica* reduced acid production in dental plaque, which could reduce development of dental caries. (Sofrata et al., 2007). As a result of these scientific evidences, extracts from *S. persica* are now incorporated into commercial preparations of a number of toothpastes worldwide (Chaurasia et al., 2013). Some of the commercially available toothpastes produced with *S. persica* extracts are Sarkan toothpaste (UK), QualiMeswak toothpaste (Switzerland), Epident toothpaste (Egypt), Siwak- F toothpaste (Indonesia), Fluoroswak, Miswak (Pakistan) and Dentacare Miswak Plus (Saudi Arabia) (Chaurasia et al., 2013).

Reports from other regions of the world have also demonstrated the potential of traditional medicinal in the control of dental caries and periodontal diseases, although extracts from a number of these plants are yet to be developed into meaningful oral care products. In the Oriental Asia, powdered leaves of *Drosera peltata* Smith, a carnivorous plant belonging to the family Droseraceae, is used by native people in the treatment of dental caries (Didry et al., 1998). Chloroform extract of the aerial part of *Drosera peltata* has been shown to have broad-spectrum activity against numerous bacteria of the oral cavity, with greatest activity against caries associated *S. mutans* and *S. sobrinus* and moderate activity against periodontal disease associated *Prevotella intermedia*. The compound Plumbagin was identified as the active component of this extract (Didry et al., 1998). A Chinese traditional medicinal plant, *Coptidis*

Rhizoma (family: Ranunculaceae), is used in treatment of many diseases, including toothache (WHO, 1999). A boiling water extract of *Coptidis rhizoma* was shown to have marked inhibitory effects on the growth of periodontopathic bacteria *Actinomyces naeslundii*, *P. gingivalis*, *P. intermedia*, *Prevotella nigrescens*, *A. actinomycetemcomitans* (MIC: 0.031-0.25 mg/ml) and less effects on all tested strains of cariogenic bacteria *Streptococcus* and *Lactobacillus* (MIC: 0.5-2 mg/ml), thus suggesting the potential of its clinical application in treatment of periodontal diseases (Hu et al., 2000). In the Mediterranean region, the plant *Helichrysum italicum (*Roth) G. Don fil. (Family: Asteraceae) has been used for its medicinal properties for a long time. The plant has many traditional uses including treatment of toothache (Viegas et al., 2014) An ethanol extract from this plant was found to exert anti-bacterial effects on cariogenic *S. mutans*, *S. sanguis* and *S. sobrinus*, with MIC values of 31.25–62.5 μgmL−1 (Nostro et al., 2004). More and co-workers studied the effects of ethanol extracts from eight South African medicinal plants used for traditional oral care and the majority of the plants showed inhibitory effects on *A. naeslundi, Actinomyces israelii, P. gingivalis, P. ntermmedia* and *S. mutans* (More et al., 2008). Based on the accumulating evidences from different regions of the world, WHO is encouraging the use of plants as a tool for oral healthcare in areas where this is customary (WHO, 1987). Additionally, the Consensus Statement on Oral Hygiene (2000) stated that medicinal plants may have a role to play in the promotion of oral hygiene, and that evaluation of their effectiveness warrants further research to clearly define their anti-microbial effects (Anonymous, 2000).

Most studies investigating anti-microbial effects of medicinal plants on oral pathogens have utilized crude plant extracts. The crude extracts are usually obtained by grinding dried plant materials, followed by macerating in suitable solvent, centrifuging and using the supernatant after filtration as the test material. We argued that this method of obtaining extracts for testing may not truly reflect the way most of these plants are used traditionally for dental or oral care, especially in situations where the plant materials are used in fresh form. For example, in Uganda and East African region, for treatment of teething syndrome, fresh leaves of *Momordica foetida, Carissa edulis*.Vahl (Mubiru et al., 1994) and *Solanum nigrum (*Kokwaro, 1993) are pounded and the resultant paste applied or squeezed onto the gum. For treatment of toothache, fresh leaves of *Ocimum gratissimum, Bidens pilosa*, (Mubiru et al., 1994) and the fresh stem bark of *Zanthoxylum chalybeum* (Kokwaro, 1993) are chewed. To treat mouth wounds, fresh leaves from *Hoslundia opposita* are chewed (Hamill et al., 2003; Kokwaro, 1993). Chewing sticks prepared from fresh stems or roots of *Vernonia amygdalina, Euclea*

latidens. Stapf, *Cymbopogon nardus* (Mubiru et al., 1994), *Zanthoxylum chalybeum* (Kokwaro, 1993) *Lantana trifolia* and *Teclea nobilis*. Delile (Hamill et al., 2003) are also used to prevent or treat dental caries. The widespread use of fresh plant parts for traditional oral care prompted part of this thesis to assess the possibility of release of anti-bacterial substances from the fresh extracts/pulp juices produced during use, and to consider whether such a release could be of significance in the prevention of dental caries or periodontal diseases (Study I).

1.3 Essential oils and oral pathogens

Among the several classes of plant chemicals that have been used for medicinal purposes are the alkaloids, glycosides, flavonoids, phenolic compounds, quinones, tannins, and terpenoids (essential oils) (Allaker and Douglas, 2009; Janardhanan and Thoppil, 2004). Of particular interest are the essential oils because they have been used for hundreds of years and are still being used as components of herbal treatment for a variety of ailments (Janardhanan and Thoppil, 2004). Essential oils are aromatic, volatile products of secondary metabolism of many plants species, and are known to play varied roles in the plants, some of which include attraction of insects and protection of the plants against harmful microbial/viral attack. They can be found in almost every plant parts including roots, stems, leaves, flowers, fruits, seeds (Janardhanan and Thoppil, 2004; Raut and Karuppayil, 2014). Essential oils are not only complex, but also chemically heterogeneous. They are derived from Isoprene (2-methyl-1,3-butadiene), a common organic compound that is widely distributed throughout the plant kingdom (Cowan, 1999). Plants producing essential oils belong to around 60 families, but only selected families like Alliaceae, Lamiaceae, Rutaceae, Myrtaceae, Apiaceae, Asteraceae, Lauraceae, Poaceae, Verbenaceae are known to produce essential oils of medicinal and industrial values (Raut and Karuppayil, 2014).

Essential oils exhibit a variety of pharmacological effects including anti-oxidant (Bhalla et al., 2013) and anti-microbial (Hammer et al., 1999; Kalemba and Kunicka, 2003) properties and have thus found considerable range of pharmaceutical applications. A number of recent studies add to the evidences that essential oils are suitable additives in products used for the maintenance of oral hygiene or prevention of dental diseases. Perhaps, the most extensively studied essential oil is that from the Australian medicinal plants *Melaleuca alternifolia* (Family: Myrtaceae). The essential oil from this plant has been used medicinally for many years. Hammer and co-workers investigated the anti-bacterial effects of *Melaleuca*

alternifolia essential oil on a collection of bacteria isolated from oral cavity and reported MIC and MBC values in the range 0.003–2.0% (v/v) (Hammer et al., 2003). Further, time-kill assays showed that exposure of *S. mutans* and *Lactobacillus rhamnosus* to 0.5% (v/v) *Melaleuca alternifolia* essential oil resulted in >3 log reduction of viable cells within 30 seconds (Hammer et al., 2003). Another study compared the anti-bacterial efficacies of *Melaleuca alternifolia* essential oil and other essential oils including manuka oil, eucalyptus oil, lavandula oil and rosmarinus oil against oral pathogens and found that, in addition to their inhibitory and bactericidal effects, most of the oils were able to inhibit the adhesion of *S. mutans* and *P. gingivalis* (Takarada et al., 2004). Another important medicinal plants in Asia is *Cryptomeria japonica* (Family: Taxodiaceae) (Cha et al., 2007). Essential oil from *C. japonica* was shown to exhibit strong anti-bacterial effects on several oral bacteria including *Streptococcus* spp., *A. actinomycetemcomitans* and *P. gingivalis* with MIC of 0.025–0.5mg/ml (Cha et al., 2007). Essential oils have also been demonstrated to enhance the effect of chlorhexidine. When used in combination with chlorhexidine, essential oils of cinnamon and manuka were able to significantly reduce the amount of chlorhexidine required to inhibit the growth of oral pathogens (Filoche et al., 2005). These studies and others indicate that essential oil from medicinal plants can be used in oral health care products and in the maintenance of oral hygiene. And for many years now, Listerine™ (Essential Oil Rinse) has been in widespread use (Allaker and Douglas, 2009; Fine, 2010). The Listerine™ product contains four plant essential oils, namely, eucalyptol, menthol, thymol and methyl salicylate (Fine, 2010).

A number of medicinal plants used for traditional treatment of oral diseases in Uganda belong to the aromatic plant families and are thus essential oils producing (Table 1). Given the potential of the essential oils in the promotion of oral health, a section of this thesis was focused on studying the inhibitory effects of essential oil from these aromatic plants on the growth of oral pathogens (Study II).

1.4 Inflammation and anti-inflammatory effects of compounds of plant origin

Inflammatory process may be defined as a sequence of events that occur in response to infection, trauma or toxic stimuli. The process of inflammation is essential for defense against pathogens and wound healing, and thus occurs throughout the human body. Acute inflammation is a type of inflammation that is of short duration, and usually occurs

immediately in response to trauma or infection. However, when the inflammation remains unresolved, it develops into chronic inflammation. This happens when the host immune and inflammatory responses are insufficient to remove or clear the microbial challenge which initiates and spreads the disease process. In chronic inflammation, tissue destruction and healing occur concurrently, but the balance is delicate and can easily shift towards destruction if environmental and certain host factors like genetic and systematic conditions favour it (Yucel-Lindberg and Bage, 2013). In the oral cavity, hundreds of species of aerobic and anaerobic bacteria are constantly present. Some of these species grow on tooth surfaces as complex, mixed colonies in biofilms (Socransky and Haffajee, 2005). Certain Gram-negative bacterial species such *Porphyromonas gingivalis, Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans* that are present in the biofilms are known to initiate an inflammatory response that result to gingivitis. In diseases-susceptible individuals, gingivitis may progress to periodontitis (Bartold and van Dyke, 2013)

The term "periodontal disease" is a collective term for the inflammatory disorders of gingivitis and periodontitis instigated by bacteria in the biofilms (Pihlstrom et al., 2005) Gingivitis is a mild, but common form of inflammation of the gingiva. Gingivitis is easily reversible by improvement in oral hygiene in healthy individuals. Periodontitis occurs when the gingival inflammation persist, and extend deep into the tissues and thus become chronic. This leads to irreversible destruction of the supporting structures of the teeth. The typical characteristics of periodontitis are destruction of the periodontal ligaments, gingival tissues and alveolar bone loss (Pihlstrom et al., 2005).

Inflammatory response in the periodontal tissues and other parts of the body are orchestrated by a complicated interaction between mediators of inflammation and inflammatory cells (Kinane et al., 2011). Cytokines are the soluble proteins that serves as mediators of cellular communication and are involved in many steps of the inflammatory response. More than 100 members of the cytokine family and their specific receptors have been identified (Haddad, 2002). Cytokines has been classified as pro-inflammatory and anti-inflammatory, depending on the way they influence inflammatory process. Pro-inflammatory cytokines (e.g IL-1β, TNf-α, IL-6 and IL-8) are known to promote the inflammatory process, whereas the anti-inflammatory cytokines (e.g IL-4, IL-10) suppress the activity of pro inflammatory cytokines (Dinarello, 2000; Opal and DePalo, 2000).

Cytokines are synthesized by nearly every cell including migrating cells (e.g masts cells, macrophages and neutrophils) as well as resident cells such as gingival fibroblasts and epithelial cells. After release; cytokines can act either locally or systemically. Due to their redundant and pleiotropic actions, cytokines function in a network in which one cytokine can induce its own production or even the secondary generation of other cytokines. Furthermore, most cytokines are known to be involve in the activation of transcription factors and protein kinases that in turn regulate the expression of many target genes that are vital for the maintenance of the inflammatory process (Kracht and Saklatvala, 2002) For instance, cytokine may be responsible for induction of several enzymes (COX-2, NO synthase, adhesion molecules for leukocyte) that augment blood flow and recruit leukocytes to the site of infection (Kracht and Saklatvala, 2002). A different group of cytokines, known as chemokine (including IL-8, MCP-3) have the ability to chemo attract and activate leukocytes at the site of inflammation (Luster, 1998; Wong and Fish, 2003). Binding of chemokine to their specific receptors allows rolling leukocytes to become firmly adherent and to transmigrate to the target tissues, a process which depends on the activation of adhesion molecules, mainly integrins (Luster, 1998).

Cytokines (and chemokines) are now known to be involved in the pathophysiology of many inflammatory diseases including periodontitis (Hopkins, 2003; Yucel-Lindberg and Bage, 2013). The pathological states in periodontitis have been linked to an imbalance of the cytokines network in the periodontal tissues following stimulation by periodontopathic bacteria in the dental biofilm and /or their products. The continuous production of the various pro-inflammatory cytokines, including interleukin (IL) 1β, IL-6, IL-8, TNF-α), as well as of prostaglandin E_2 (PGE_2) following this stimulation had been associated with periodontal tissue destruction (Okada and Murakami, 1998).

The Consensus of the Seventh European Conference on Periodontology underscored the need to develop new and more effective preventive and treatment approaches for periodontal diseases which embraces inflammation resolution as well as direct management of micro biota. (Tonetti and Chapple, 2011). Compounds that are able to selectively interfere with production and/or functions of cytokines, thus bringing about resolution of inflammation in the periodontal tissues would constitute an important alternative for treatment of periodontal diseases (Tonetti and Chapple, 2011). In an effort to identity such compounds, part of this

thesis was devoted to investigation of the effects of essential oils from the Uganda medicinal plants on the production of pro-inflammatory cytokine (IL-6, IL-8 and prostaglandin E2) by human gingival fibroblasts. The essential oils that were investigated were those with promising anti-bacterial effects on periodontopathic bacteria (Study III).

The potential effects of essential oils from medicinal plants on production of inflammatory cytokines/mediators have previously been reported by other authors. Tipton and co-workers found essential oil from *Commiphora molmol* (myrrh oil) to significantly reduced IL-β stimulated production of IL-6 and IL-8 by gingival fibroblasts cells (Tipton et al., 2003). The water soluble fraction of essential oil of *Melaleuca alternifolia* (tea tree oil) was found to inhibit lipopolysaccharide induced production of the inflammatory mediators TNF-α, IL-β and IL-10 by human peripheral blood monocytes approximately 50% and that PGE_2 by about 30% (Hart et al., 2000).

1.5 Toxicity testing of medicinal plants

Plants used in traditional medicine are assumed to be safe. This safety is based on their long usage in the treatment of diseases according to knowledge accumulated over centuries (Fennell et al., 2004). However, a study carried out to investigate potential hazards associated with long –term effect of 51 plants commonly used in South African traditional medicine found that most of these plants either caused DNA damage, chromosomal aberration or chromosomal lagging in human white blood cells (Taylor et al., 2003). It cannot therefore be taken for granted that medicinal plants are void of toxic effects. Some authors have thus recommended that pharmacological studies on medicinal plants should always be followed by toxicological screening (Cos et al., 2006).

Toxicity may be tested in-*vivo* using animals such as rats, guinea pigs, monkeys, or in-*vitro* using cell lines. In-*vivo* animal models are usually used to provide evidence of systemic or organ toxicity. In *vitro* cytotoxicity testing of medical/dental materials on host cells is usually recommended before commencement of other advanced systemic or organ toxicity tests (ISO, 2009). As a first step towards other advanced tests, we tested the cytotoxicity of selected plant extracts using human gingival fibroblasts model (Study III). The human gingival fibroblasts are suitable model because they are the predominant connective tissue

cells (65 % of the total cell population) of the periodontal tissue and are exposed to the plant extracts during use.

2 AIMS OF THE THESIS

2.1 Overall aim

The overall aim of this thesis was to investigate the Ugandan medicinal plants that are used traditionally for oral care for anti-bacterial, cytotoxic and anti-inflammatory effects.

2.2 SPECIFIC AIMS

i. To test the anti-bacterial effects of fresh pulp juice obtained from 16 commonly used plant species on cariogenic and periodontopathic bacteteria..
ii. To test the anti-bacterial effects of solvent extracts (water, hexane, methanol) obtained from the 16 commonly used plant species on cariogenic and periodontopathic bacteteria.
iii. To test anti-bacterial effects of essential oils from selected aromatic medicnal plants on cariogenic and periodontopathic bacteteria.
iv. To test the cytotoxic effects of the promising plant extracts on the human gingival fibroblasts.
v. To test the effects of the promising extracts on the production of pro-inflamatory cyotkines.
vi. To identify the bioactive molecules in the most active extracts.

3 MATERIALS AND METHODS

3.1 Plant materials used in the study and their collection

The plant materials used in this study were collected from the regions of Uganda where they are used traditionally for treatment of various form of dental and oral diseases. The plants were collected following information obtained from literature. Collection was carried out in consultation with the local people or traditional healers in the areas, and local names of the plants got from literature were used to aid identification. The plants were collected in 2 parts: the first part was used for scientific authentication; the second part was for experimental use. The first part, which consisted of a representative sample (i.e. stem, leaves, flowers and fruit, if any) was pressed between two boards and taken to the botanist at the Herbarium, Department of Botany, Makerere University for scientific authentication. The specimens were archived at the Herbarium at the Department of Botany, Makerere University after voucher specimen numbering. The following information were included in the voucher of each specimen: Voucher number, confirmed scientific name, local plant name, dental/oral diseases treated, district of collection, county, sub-county, parish, village, habitat and date of collection. The 16 medicinal plants collected are shown in Table1. The second part of plants collection included parts of the plant that are used traditionally and the collection was handled depending on the experiment that was to be carried out. Plants destined for extraction of fresh pulp juice or essential oils were transported in ice-cooled boxes and stored at -80 ^{o}C at the Department of Medical Microbiology, Makerere University, until use or transportation to Karolinska Institutet, Sweden. Schematic describing the studies done on the medicinal plants is shown in Figure 1.

Table 1. Uganda medicinal plants used for traditional treatment of dental/oral diseases

Scientific name/plant family/voucher number[a]	Local name/tribe	District of collection	Plant part: Ethno-medical use(s) (references)	Dental/oral disease(s) treated
Carissa edulis.Vahl (APOCYNACEAE) [FO-001]	Achuga/Langi	Apach	LT: pound, add cold water and squeeze few drops on gum (Mubiru et al., 1994)	Teething syndrome
Bidens pilosa (ASTERACEAE) [FO-002]	Enyabarashana/Rukiga	Kabale	LT: chew (Mubiru et al., 1994)	Toothache
Crassocephalum vitellinum (ASTERACEAE) [FO-003]	Paroti / Kupsabiny	Kapchorwa	LT: Dry, burn and rub ash on false teeth (Mubiru et al., 1994)	Teething syndrome
Helichrysum odoratissimum (ASTERACEAE) [FO-004]	Chebushe / Kupsabiny	Kapchorwa	LT: Dry, burn and rub ash on false teeth (Mubiru et al., 1994)	Teething syndrome
Vernonia amygdalina (ASTERACEAE) [FO-005]	Okello-okello/Langi	Apach	ST: use to brush teeth (Mubiru et al., 1994)	Dental caries
Euclea latidens. Stapf (EBENACEAE) [FO-006]	Amuru-dyek/Langi	Apach	RT: Use to brush teeth (Mubiru et al., 1994)	Dental caries
Momordica foetida K.Schumach (CUCURBITACEAE) [FO-007]	Bomo/Langi	Apach	LT: Pound, squeeze juice onto the wound (Mubiru et al., 1994)	Teething syndrome
Hoslundia opposita (LAMIACEACE) [FO-008]	Kamunye/Baganda	Masaka	LT: Chew (Hamill et al., 2003; Kokwaro, 1993)	Mouth wounds
Ocimum gratissimum (LAMIACEACE) [FO-009]	Omujaja/Rukiga	Kabale	LT: Chew (Mubiru et al., 1994)	Toothache
Cymbopogon citratus (POACEAE) [FO-010]	Kasubi/Baganda	Kampala	LT: taken as tea (Mubiru et al., 1994)	Bad breath
Cymbopogon nardus (POACEAE) [FO-011]	Omute/Rukiga	Kabale	Root: to brush teeth (Mubiru et al., 1994)	Bad breath, dental caries.
Clematis hirsuta. Guill. & Perr. (RANUNCULACEAE) [FO-012]	Adwe/Langi	Apach	LT: pound and apply paste on painful tooth, Infusion drunk (Kokwaro, 1993)	Toothache Sore throat
Teclea nobilis. Delile (RUTACEAE) [FO-013]	Nzo/Baganda	Rakai	ST: Use to brush teeth (Hamill et al., 2003)	Dental caries
Zanthoxylum chalybeum (RUTACEAE) [FO-014]	Owucu/Langi Songowowo/Pokot	Apach	RT: Use to brush ST: Bark chewed (Kokwaro, 1993)	Dental caries Toothache
Solanum nigrum (SOLANACEAE) [FO-015]	Osuga/Langi	Apach	LT, FT: crush into paste and rub on the gum (Kokwaro, 1993)	Teething syndrome
Lantana trifolia (VERBENCAEACE) [FO-016]	Kayuki-yuki/Baganda	Masaka	ST: Use as toothbrush to clean and freshen LT: infusion swallowed (Hamill et al., 2003; Odongo et al., 2011)	Oral Hygiene Tonsillitis

[a] Voucher specimen number at the Herbarium, Makerere University; FT=Fruit; LT=Leave; RT=Root; ST=Stem.

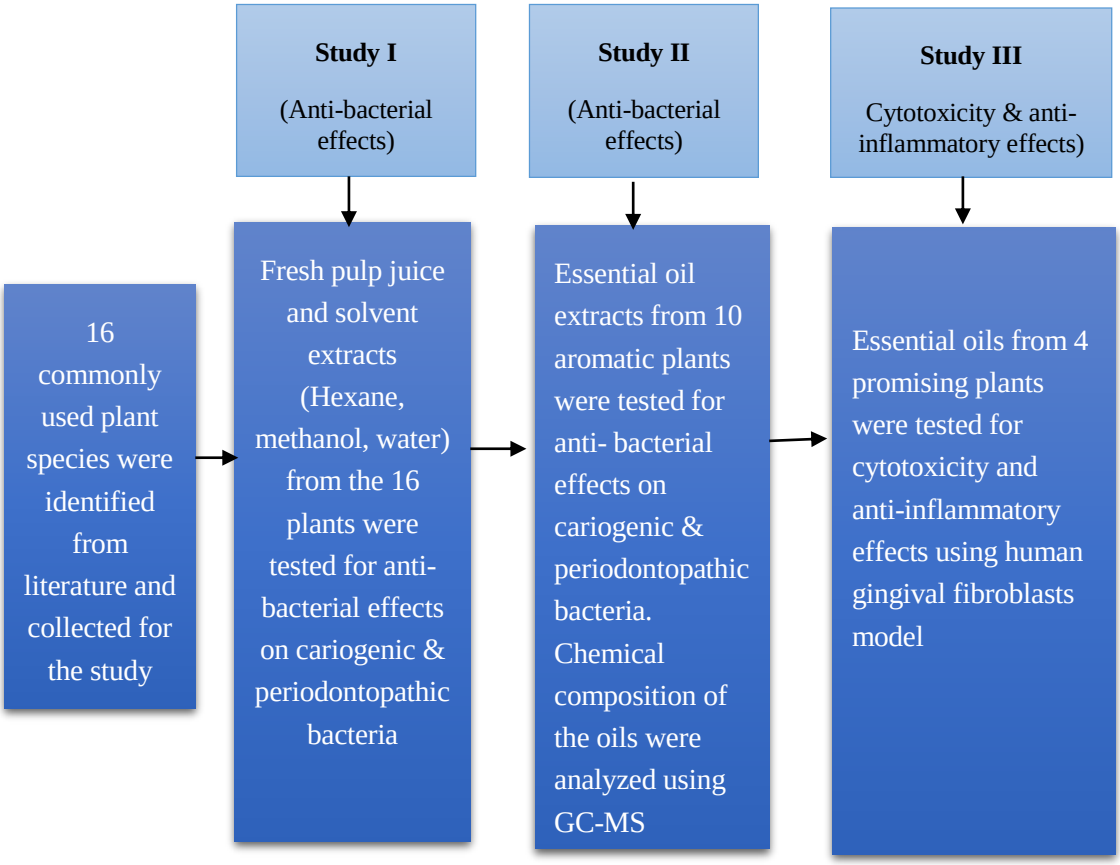


Figure 1. Schematic describing the studies done on the medicinal plants

3.2 Study I

3.2.1 Preparation of fresh pulp juice

To obtain fresh pulp juice for testing, fresh plant materials (stored at -80 ^{o}C) were thawed, rinsed with distilled water, and routinely 50 g cut into small pieces and a manual fruit juicer (Anjali fruit juicer, JL01, Mumbai, India) (Figure 2) used to crush and express the juice from the fresh plant pieces. The expressed juice was prepared for testing immediately after preparation at full strength (100%), 50% and 25% dilutions. The dilutions were made using brain heart infusion (BHI) broth.

Figure 2. Anjali fruit juicer

3.2.2 Preparation of solvent extracts

Solvent extracts for the study were obtained from dry plant materials. For woody plant species (trees and shrubs), the stem/stem bark, leaves, and roots were collected, processed and later tested separately, while for herbaceous plant species, the aerial parts consisting of leaves and stems were processed together. The preparation method of plant materials for solvent extract was adapted from Matu and van Staden (2003) with modifications. The plants were first dried at room temperature for a period of 2-3 weeks, and then milled into powder with a Brook Crompton Series 2000 mill (Huddersfield, UK) at the Natural Chemotherapeutics Research Laboratories (NCRL), Kampala, Uganda. The powders were packed in re-sealable plastics bags and stored in the dark at room temperature until solvent extraction.

In order to extract all possible active compounds present in the plant, three solvents of increasing polarity were used. The solvents used were: n-hexane (non-polar), methanol (polar) and water (very polar). Hexane and methanol extracts were obtained by routinely macerating 300-500 g of the powdered materials in approximately 600-2000 mls of hexane or methanol, with occasional shaking for 72 hours, and filtered using Whatman grade 1 filter paper. The filtrate was concentrated using a rotary vacuum evaporator (Heidolph, Germany) to approximately 50 mls and the resultant concentrate dried in the oven at 40-50 ^{o}C until weight remain constant. The extract was stored at 4 ^{o}C until use. For the water extract, 10-35 g of the powdered plant material was heated in 200-500 mls of distilled water to boiling point for about 20 minutes and the extract filtered using Whatman grade 1 filter paper. The filtrate was concentrated using a freeze dryer and the residue stored in re-sealed plastic bags at 4 ^{o}C until use.

3.2.3 Bacterial strains and cultivation

3.2.3.1 Bacteria strains

The bacterial strains that were used in study I were obtained from the culture collection of the University of Goteborg (CCUG). The strains used include the dental caries associated *Streptococcus mutans* (CCUG 6519 T), *Streptococcus sobrinus* (CCUG 25735 T), *Lactobacillus acidophilus* (CCUG 5917 T)**,** and the periodontal diseases associated *Porphyromonas gingivalis* (CCUG 25226), *Aggregatibacter actinomycetemcomitans* (CCUG 56173) and *Tannerella forsythia* (CCUG 21028 AT)**.**

3.2.3.2 Bacterial cultivation

Growth conditions: The bacteria were cultivated as previously described (Sofrata et al., 2008) but with modifications. *S. mutans* was grown for 2 days under anaerobic conditions on Colombia base agar supplemented with 5% sheep blood. *L. acidophilus* was grown for 2 days under anaerobic conditions on Colombia base agar supplemented with 5% sheep blood. *A. actinomycetemcomitans* was grown for 2 days under anaerobic conditions on Colombia base agar supplemented with 5% sheep blood and incubated in 95% air and 5% carbon dioxide. *P. gingivalis* was grown anaerobically for 4 days on Colombia base agar supplemented with hemin (0.05mg/ml), vitamin K (0.01mg/ml) and 5% sheep blood. *T. forsythia* was grown anaerobically in chocolate agar supplemented with hemin (0.05mg/ml) and vitamin K (0.01mg/ml).

3.2.4 Anti-bacterial activity testing

3.2.4.1 Fresh pulp juice

A simple agar well-diffusion assay was used to test for anti-bacterial activities in the fresh pulp juice. For testing, *S. mutans, S. sobrinus, L. acidophilus* and *A. actinomycetemcomitans* were suspended in phosphate buffered saline (PBS) and *P. gingivalis* and *T. forsythia* were suspended in peptone yeast glucose. The density of each bacterial suspension was adjusted using a spectrophotometer to equal that of 0.5 McFarland standards (10^8 colony forming units/ml). Each bacterial suspension was swabbed over the surface of their respective special agar plate prepared as described in section *3.2.3.2* above and thereafter a 7 mm core of agar

was removed from three positions on each plate (Figure 3). The wells were aseptically filled as follows: first well was filled with 0.1mls of fresh pulp juice or dilutions; the second with 0.1mls of doxycycline (30µg) solution in normal saline to act as positive control; the third with 0.1 mls of BHI broth to act as negative control. After holding the plate for 1 hour, the plates were incubated under the conditions described in section *3.2.3.2* above followed by the measurement of inhibition zones in millimeters. The experiments were repeated three times and the anti-bacterial activity was expressed as the ratio of the inhibition zone produced by plant extract and the inhibition caused by the doxycycline control in the plate (Vlietinck et al., 1995).

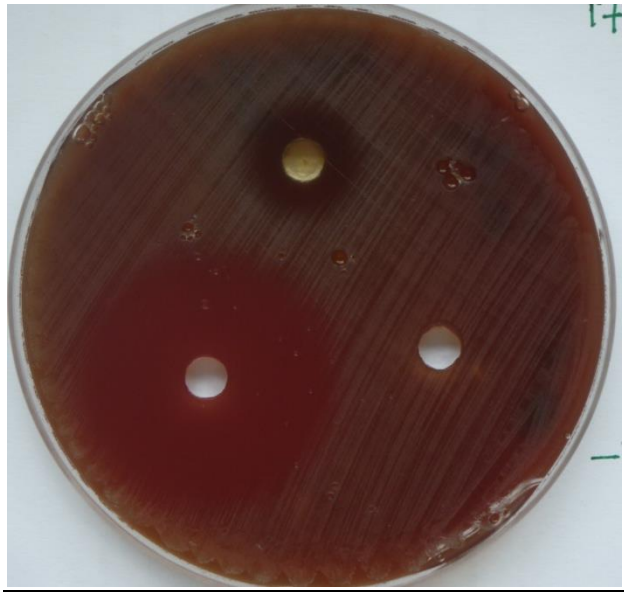


Figure 3. Positions of well in the agar plate and inhibition zones

3.2.4.2 Solvent extracts

A total of 66 solvent extracts from 15 plant species were prepared for testing. One plant species (*Carissa edulis*) could not be obtained for solvent extraction. Screening of the extracts for anti-bacterial activity was done using agar-dilution assay (Rios et al., 1988) and the special media prepared for the bacteria in section *3.2.3.2* above were used. The extracts were first screened for activity at a maximum concentration of 1mg/ml as recommended for crude plant extracts (Cos et al., 2006) and if active, the minimum inhibitory concentrations (MIC) then determined following the European Committee for Antimicrobial Susceptibility Testing (EUCAST) protocol for determination of MIC of anti-bacterial agents by agar dilution (EUCAST, 2000) with modifications. For testing at a maximum concentration of 1mg/ml, 0.5mls of the extract at a concentration of 20mg/ml in 10% dimethyl sulfoxide (DMSO) or PBS (for water extract) was added to 9.5mls of melted agar held at 45-50 ^{o}C in a falcon tube, shaken and poured in

petri-dish to harden. The final in-test concentration of DMSO in the agar was 1% (v/v). Preliminary tests had shown this final DMSO concentration not inhibitory to bacterial growth. The bacteria were suspended as described in section *3.2.4.1* above and each suspension adjusted to give 10^7 CFU/ml. Using a micropipette, 1µl of the suspension (10^4 live bacterial cells) was spotted onto the surface of agar plates and the plate allowed to stand until the inoculum was completely absorbed (EUCAST, 2000). A plate with 1% DMSO (solvent control) and another incorporated with 10µg doxycycline were also inoculated to serve as positive and negative controls respectively. The plates were incubated anaerobically at 37 ^{o}C for 24 to 48 hours. The extract was declared active if it completely inhibited visible growth of bacteria as judged by the naked eye, disregarding a single colony or a thin haze within the area of the inoculated spot (EUCAST, 2000)

For MIC determination, two fold dilutions of the extracts in DMSO or PBS (for water extract) were prepared at final in-test concentrations ranging from 0.0313 to 1 mg/ml, and the bacteria prepared and spotted as above. The MIC of doxycycline for each bacterium was also run to validate the procedures. The final in-test concentrations of doxycycline ranged from 7.8×10^{-5} to 2.5×10^{-3} mg/ml. MIC was the lowest concentration of the extract that completely inhibited visible growth of bacteria as judged by the naked eye, again disregarding a single colony or a thin haze within the area of the inoculated spot (EUCAST, 2000). All tests were performed in duplicates

3.2.5 Graphical summary of study I

Graphical summary of study I is shown in Figure 4

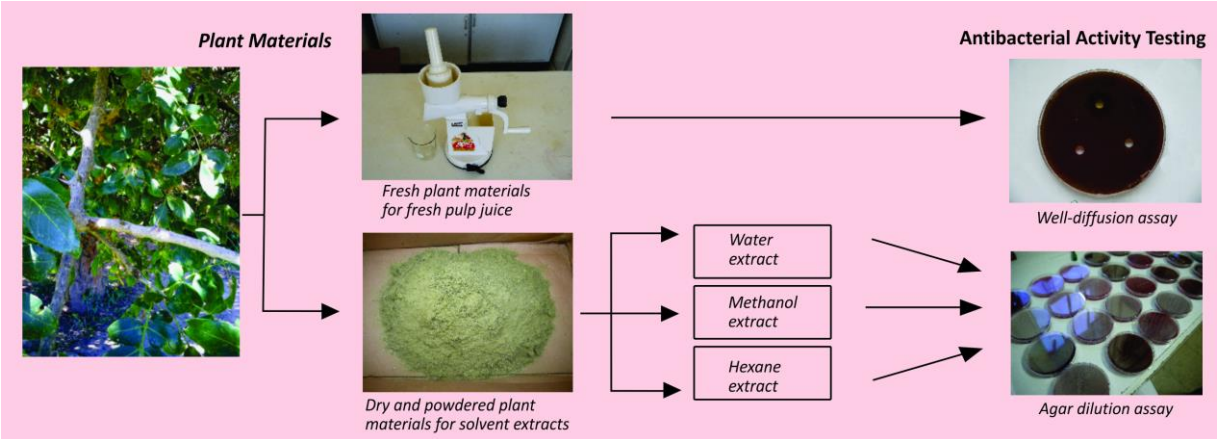


Figure 4. Graphical summary of study I

3.3 Study II

3.3.1 Plant materials for extraction of essential oils

In study II, ten aromatic plants were selected for extraction of essential oils. The ten plants selected were *B. pilosa, H. odoratissimum, V. amygdalina, H. opposita, O. gratissimum, C. citratus, C. nardus, T. nobilis, Z. chalybeum* and *L. trifolia*. These plants were selected from the original 16 plants cited in Table 1 on the basis of their grouping in the aromatic plant families (Raut and Karuppayil, 2014; Skaria et al., 2007). The aromatic plants families from where the species were picked include Asteraceae, Lamiaceae, Poaceae, Rutaceae, and Verbenaceae.

3.3.2 Extraction of essential oil

To extract essential oil, 300g of cut pieces of fresh plant material was mixed with 600 ml of distilled water and the mixture subjected to hydro-distillation for 4-5 hours using glass distillation apparatus as shown in Figure 5. The distillate (oil/water mixture) was collected, and the essential oil extracted from the distillate with HPLC grade hexane using a separating funnel. Anhydrous magnesium sulphate was added to the hexane extract to remove any trace of water. After filtration, hexane was evaporated with a rotary evaporator (Rotavapor-R210, Buchi, Switzerland). The essential oil obtained from each plant was weighed and the yield calculated as percentage of fresh starting plant material.

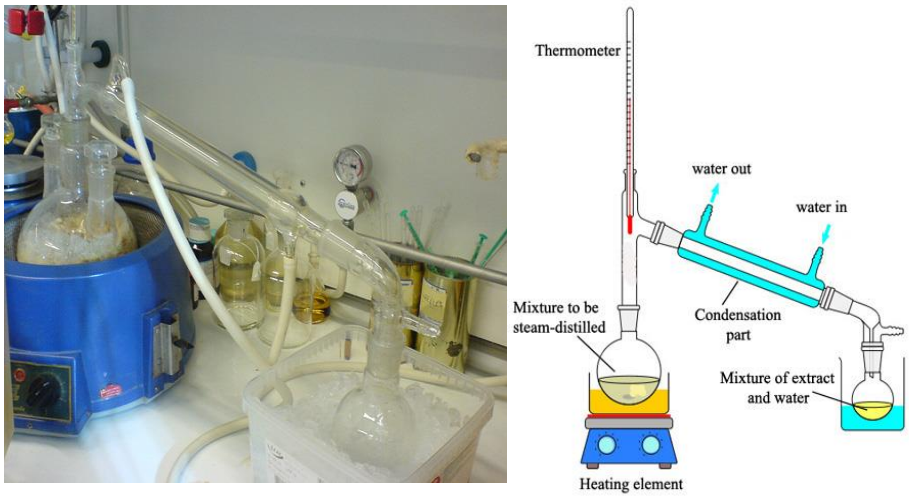


Figure 5. Steam distillation set-up.

3.3.3 Bacterial strains and cultivation

3.3.3.1 Bacterial strains

Gram-negative bacterial strains: Periodontopathic bacteria *A. actinomycetemcomitans* (HK 1519) and *P. gingivalis* (ATCC 33277).

Gram-positive bacterial strains: Cariogenic bacteria *S. mutans* (CCUG 27624) and *L. acidophilus* (NCTC 1723) and the non-oral pathogenic bacterium *Bacillus megaterium* (BM11).

3.3.3.2 Bacterial cultivation

Growth conditions: All the bacteria were propagated as previously described (Sofrata et al., 2011). *A. actinomycetemcomitans* was propagated on Columbia base agar supplemented with 0.1% tryptophan and 5% citrated horse blood in 5% CO_2 atmosphere. *P. gingivalis* was propagated for 6 days on Colombia base agar supplemented with hemin (0.05mg/ml), vitamin K (0.01mg/ml) and 5% citrated horse blood in an anaerobic atmosphere. *S. mutans* was grown in Brain Heart Infusion (BHI) agar plates for 2 days in 5% CO_2 atmosphere. *L. acidophilus* was propagated for two days on Lactobacilli MRS agar plates in 5% CO_2. *B. megaterium* was propagated overnight in air on Luria agar plates. All bacterial were incubated at 37 ^{o}C.

3.3.4 Analysis of chemical composition of essential oils

The chemical composition of the essential oils were analyzed using a Varian 3400 Gas-Chromatography (GC) connected to a Finnigan SSQ 7000 Quadropole Mass Spectrometer (MS). The GC was equipped with a split/splitless injector (splitless mode 30 seconds), a DB-wax capillary column (J & W Scientific, Folsom, CA, and USA, 30m long, 0.25mm inner diameter and 0. 25µm film thickness). The injection temperature was isothermally set at 230 ^{o}C. The carrier gas (Helium, 99.99%, Stransmollen AB, Ljungby, Sweden) was delivered at a constant pressure of 10 psi. A representative temperature program was 40 ^{o}C for 1 minute, followed by an increase in temperature at a rate of 3 ^{o}C /minute up to 235 ^{o}C, and thereafter the temperature was maintained at 235 ^{o}C for 14 minutes, making a total analysis time of 80 minutes. Transfer line temperature was kept at 235 ^{o}C and the MS ion source temperature was 150 ^{o}C. Mass spectra were obtained for 70 eV with a mass range of 30 to 600m/z in positive mode. The software program, X-calibur 2.0 was used for acquiring and analysis of the GC-MS data. For analysis, dried samples of essential oils were redissolved in hexane to a concentration of 5 µg/µl and 1µl injected into the GC. Identification of compounds in the

oils were made by comparison of their MS with compounds in the Finnigan NIST library-2008 and final authentication of selected compounds made by analyzing available compounds at the same parameters as those used for the essential oils.

3.3.5 Assessment of growth inhibitory effects of the essential oils

Assessment of growth inhibitory effects was done using broth dilution assay as previously described (Sofrata et al., 2011), with modifications. Colonies of *A. actinomycetemcomitans* and *P. gingivalis* were re-suspended in Peptone Yeast Glucose medium. *S. mutans* colonies were re-suspended in BHI broth. Colonies of *L. acidophilus* were re-suspended in Lactobacilli MRS broth. Colonies of *B. megaterium* were re-suspended in Luria broth. The optical densities of all bacterial suspensions were adjusted to 0.5 at λ590nm wavelength. All bacteria were further diluted in fresh growth medium 10^4 fold prior to the test. The bacterial suspensions were incubated for 90 minutes in their respective growth medium at 37 ºC in the presence of different concentrations of essentials oils. Dilutions of essential oils were prepared in dimethyl sulfoxide (DMSO) and 5µl of each dilution or undiluted oil was added to 495µl of respective growth medium to obtain final concentrations of 1%, 0.1% and 0.01%. To the solvent control, 5µl of DMSO was added. Chlorhexidine was used a positive control, diluted to final concentrations of 1%, 0.02% and 0.05% and tested against clinically relevant bacteria *A. actinomycetemcomitans, P. gingivalis, S. mutans, L. acidophilus*). After 90 minutes, the bacterial suspensions were spread on agar plates with respective growth medium and grown as in section *3.3.3.2* above. The number of live bacteria was determined by counting the colonies on each plate, which equals colony forming units (CFU). Growth inhibitory effects was expressed as the percentage of the colony forming units (CFU) in the presence of plant essential oils or chlorhexidine to the CFU in the control plate ((CFU in test / CFU in control) × 100). All tests were done in duplicates and repeated twice.

3.3.6 Data analysis

Data anaylsis was performed using Prism 6 (GraphPad Software, San Diego, CA, USA). Independent Student's t-test was used to analyze for statistical differences in CFU in the control plate and CFU in each tested concentration of the oil or chlorhexidine. Values of $p < 0.05$ were regarded as significant (n=4).

3.4 Study III

3.4.1 Plant essential oil

The essential oils used in study III were selected from the oils which were investigated in study II. Essential oils from the following plants were selected: *B. pilosa, C. nardus, Z. chalybeum* and *O. gratissimum*. The oils were selected because they had shown good anti-bacterial effects on the Gram-negative periodontopathic bacteria *A. actinomycetemcomitans* and *P. gingivalis*.

3.4.2 Chemicals

The following chemicals were used in study III: Dulbecco's Modified Eagle's Medium (DMEM), Phosphate Buffer Saline (PBS) (without calcium and magnesium), Fetal Bovine serum (FBS), trypsin (0.25%), Penicillin-Streptomycin-Glutamine (50 mg⁄mL) were purchased from Invitrogen Life Technologies (Paisley, UK). 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) Cell Viability Assay Kit was purchased from Abnova Corporation (Taipei, Taiwan). Human IL-6 and IL-8 Douset enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D systems (Minneapolis, MN, USA). Prostaglandin E_2 monoclonal enzyme immunoassay (EIA) kit was obtained from Cayman Chemicals (Ann Arbor, MI, USA).

3.4.3 Gingival biopsies

In this thesis, human gingival fibroblast cells were used to test for cytotoxicity of the essential oils and the effects of the oils on production of pro-inflammatory mediators. The human gingival fibroblast cells used were originally established from gingival biopsies obtained from 7 systemically and periodontal healthy donors (aged 7–12) with approval of the Ethical Committee at the Huddinge University Hospital, Stockholm. The biopsies from the gingival tissues were taken during dental surgical procedures that was part of a planned patient care treatment and patients were therefore not exposed to any other additional discomfort. The patients gave their informed consent for their gingival biopsies to be used for establishment of primary gingival fibroblasts cells that were used in this research

3.4.4 Gingival fibroblast cell culture

For the experiments, the gingival fibroblast cells were cultured in DMEM supplemented with 5% FBS and 1% Penicillin-Streptomycin-Glutamine (50 mg⁄mL), grown as monolayer cultures in corning cell culture flask, 75 cm2, (Nunc^{TM}, Roskilde, Denmark) and incubated at 37 ^{o}C and 5% CO_2. The media was replaced every 3 to 4 days until about 80% confluence was reached followed by detachment with 0.025% trypsin for experimental use. Cells at passages 10-15 were used in all experiments to ensure stability.

3.4.5 Cytotoxicity assay

The cytotoxic effects of the essential oils on the human gingival fibroblast cells was assessed by estimating the viability of the cells after exposing the cells to essential oils dissolved in ethanol. Cell viability was determined by MTT assay. The assay is based on the reduction of yellow tetrazolium MTT by the mitochondrial succinate dehydrogenase enzymes to insoluble formazan-blue crystals. Only viable cells with active mitochondria reduce significant amounts of MTT and the value of absorbance obtained by the plate reader is directly proportional to viability of the cells (Mosmann, 1983). The MTT Cell Viability Kit purchased from Abnova (Taipei, Taiwan) was used according to the manufacturer's instruction.

For the experiment, the human gingival fibroblast cells were seeded at a density of 10,000 cells per well in in 96-well tissue culture plates (VWR International, Leuven, Belgium) and incubated for 48 hours to allow cell adherence and cells to grow to the exponential phase of growth. The medium was then removed and cells washed twice with serum-free medium and thereafter incubated in serum –free medium with increasing concentrations of the oils solubilized in ethanol. The final maximum concentration of ethanol in medium was 0.55% and this was not cytotoxic to cells. After 24 hours, 100µl of MTT solution was added and plates were incubated for 4 hours at 37°C. The formazan crystals formed were solubilized with solubilizing solution and the absorbance determined at 620nm by a microplate spectrophotometer (Labsystem Multiskan MS, Helsinki, Finland). The average of the blank control was determined and the amount subtracted from all absorbance values. The concentration at which 50% of the cell were killed (IC_{50}) for each essential oil was determined as recommended in the MTT kit. For each essential oil, the experiment was run in triplicates

and repeated three times and the average IC_{50} calculated. The lower the IC_{50} value, the more toxic the oil, because less is required to achieve killing of the cells.

3.4.6 Measurement of IL-6, IL-8 and PGE_2

For measurement of IL-6, IL-8 and PGE_2, gingival fibroblasts cells were seeded in 24 –well plate at a density of 40,000 cells per well (in 300µl medium) and incubated for 24 hours. The medium was removed and cells rinsed twice with serum-free medium and thereafter stimulated with 300 pg/ml IL-β alone or 300pg/ml IL-β with increasing sub-cytotoxicity concentrations of oils and incubated for further 24 hours. The supernatants was collected and stored at -80^{O}C until measurement of IL-6, IL-8 or PGE_2 levels. The amount of IL-6 and IL-8 in the supernatants was determined using the Douset ELISA kit (R&D systems, Minneapolis, MN, USA). The amount of PGE2 was determined using the PGE_2 monoclonal EIA kit (Cayman Chemicals, Ann Arbor, MI, USA). The kits were used according to the manufacturers' instructions. The experiment was performed in triplicates and repeated three times.

3.4.7 Data Analysis

The statistical analyses in study III were performed using Prism 6 (GraphPad Software, San Diego, CA, USA). Sigmoidal dose responses and non-linear regression analyses were undertaken to identify the IC_{50} (concentration that causes a reduction by half of the activity of mitochondria1 dehydrogenase) values of each essential oil. To evaluate differences in IC_{50} of the essential oils and effects of essential oils on the IL-1β induced production of pro-inflammatory cytokine (IL-6, IL-8 and prostaglandin E_2), one-way ANOVA combined with Tukey's post hoc test was used. Values of $p < 0.05$ were regarded as significant.

4 RESULTS

4.1 STUDY 1

4.1.1 Fresh pulp juice

Fresh pulp juice prepared from 16 medicinal plants (10 families) used for traditional treatment of various forms of oral and dental diseases (Table 1) were screened for anti-bacterial activities against six different dental bacteria, namely, *S. mutans, S. sobrinus, L. acidophilus* and *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythia*. The pulp juice were tested at full strength without dilutions (100%), and at 50 % and 25% strengths. Fresh pulp juice from three plant species, namely *Z. chalybeum*, *E. latidens,* and *O. gratissimum* exhibited anti-bacterial activity against one or more bacterial species. The results from the anti-bacterial activity studies from the fresh pulp juice showing positive results are presented in Table 2. Fresh pulp juice from *Z. chalybeum* and *E. latidens* showed activity against all the bacterial species tested and at all strengths. The most active fresh pulp juice was from Z. *chalybeum*. It exhibited the best activity against *L. acidophilus* with the undiluted fresh pulp juice and 25% strength producing 63% and 45% zones of inhibition respectively, compared to the doxycycline control. Fresh pulp juice from *O. gratissimum* exhibited mild anti-bacterial activities on two bacterial species, *A. actinomycetemcomitans* and *T. forsythia*, at 100% and 50% strengths.

Table 2. Anti-bacterial activity of fresh pulp juice from the Ugandan medicinal plants[a]

Botanical name	**Plant Part tested**[b]	**Juice strenght**[c]	**Anti-bacterial activity**[d]					
			A.a[e]	P.g	T.f	S.m	S.s	L.a
Euclea latidens. Stapf	Root bark	100	35	34	35	35	34	56
		50	32	31	32	30	32	51
		25	26	26	29	27	24	42
Ocimum gratissimum	Leave	100	27	-	33	-	-	-
		50	21	-	26	-	-	-
		25	-	-	-	-	-	-
Zanthoxylum chalybeum	Root bark	100	52	42	38	48	37	63
		50	47	31	32	39	33	52
		25	41	23	26	32	27	45

[a]The results are the mean of three replicates and only active plants shown.
[b]The part traditionally used for treating dental disease was selected for testing
[c] Fresh pulp juice tested at 100%, 50% and 25% strengths.
[d]Anti-bacterial activity expressed as percentage of inhibition zone of fresh pulp juice to the inhibition zone of the doxycycline (30µg) control.
[e] Microorganisms tested: A.a =*Aggregatibacter actinomycetemcomitans*; P.g = *Porphyromonas gingivalis*; T.f = *Tannerella forsythia*; S.m= *Streptococcus mutans*; S.s =*Streptococcus sobrinus*, L.a= *Lactobacillus acidophilus*
– No inhibition zones

4.1.2 Solvent extracts

Extracts from 15 plants species were screened for anti-bacterial activities. Hexane, methanol, water extracts were prepared from leaves, stems, root bark or whole plant. Altogether, a total of 66 extracts were screened. The extracts were first screened at maximum concentration of 1mg/ml, and if found active, the MIC was then determined. The MIC of active extracts are summarized in Table 3. Hexane extract from the aerial part of *H. odoratissimum* exhibited the best activity with MIC ranging from 0.0125 to 0.5mg/ml on all the bacteria tested except *A. actinomycetemcomitans*. Methanol extract from *L. trifolia* was active on all bacteria tested with MIC ranging 0.25 to 1mg.ml. *L. acidophilus* was sensitive to plant extracts with all three solvents. The MIC of doxycycline (run concurrently to validate the procedures) ranged from 1.56×10^{-4} to 12.5×10^{-4} mg/ml.

Table 3. Minimum inhibitory concentrations of solvent extracts with anti-bacterial activity

Plant species	Plant part/E[a]	MIC (mg/ml)					
		A.a[b]	**P.g**	**T.f**	**S.m**	**S.s**	**L.a**
Crassocephalum vitellinum	AE/H		1				
Helichrysum odoratissimum	AE/H		0.125	0.5	0.25	0.125	0.125
Helichrysum odoratissimum	AE/M		0.5		1	1	0.5
Vernonia amygdalina	LF/M	1					
Euclea latidens	SB/M						0.5
Momordica foetida	RT/M						1
Momordica foetida	RT/W						0.5
Hoslundia opposita	LF/M						1
Hoslundia opposita	ST/W						1
Cymbopogon citratus	AE/W						1
Cymbopogon nardus	AE/M	0.5	1	1			0.5
Teclea nobilis	LF/H		1				
Zanthoxylum chalybeum	LF/W						1
Zanthoxylum chalybeum	SB/H	0.5					1
Zanthoxylum chalybeum	SB/W						1
Zanthoxylum chalybeum	RB/H	1		1			1
Zanthoxylum chalybeum	RB/M	0.5	0.5	1		1	1
Lantana trifolia	LF/H	0.5	0.5		1		
Lantana trifolia	LF/M	0.25	1	0.5	1	1	1
Lantana trifolia	LF/W	1					
Doxycycline		1.56×10^{-4}	3.13×10^{-4}	6.25×10^{-4}	1.56×10^{-4}	3.13×10^{-4}	12.5×10^{-4}
DMSO 1% (Solvent Control)		-	-	-	-	-	-

[a] Active plant part/E: AE =Aerial part; LF =Leaves; SB=Stem bark; ST= Stem; RT= Root; RB= Root bark; H=Hexane extract; M=Methanol extract ; W=Water extract.

[b] Microorganisms: A.a =*Aggregatibacter actinomycetemcomitans*; P.g = *Porphyromonas gingivalis*; T.f = *Tannerella forsythia*; S.m= *Streptococcus mutans*; S.s =*Streptococcus sobrinus*, L.a= *Lactobacillus acidophilus*

4.2 STUDY II

4.2.1 Essential oil yields

The essential oils were extracted from 10 aromatic plants and the yields obtained determined. The essential oil yields expressed in relation to fresh weight of plant materials (% w/w) are presented in Table 4. The yields varied from 0.05% to 0.39%. The highest yield (0.39 % w/w) and the lowest (0.05% w/w) were obtained from *C. citratus* and *B. pilosa* respectively.

Table 4. Essential oil yields obtained from the plant species

Plant species	Essential oil Yield (% w/w)
Bidens pilosa	0.05
Helichrysum odoratissimum	0.31
Vernonia amygdalina	N.D[a]
Hoslundia opposita	0.12
Ocimum gratissimum	0.21
Cymbopogon citratus	0.39
Cymbopogon nardus	0.36
Teclea nobilis. Delile	0.16
Zanthoxylum chalybeum	0.21
Lantana trifolia	0.14

[a]N.D= yield not determined

4.2.2 Chemical composition

The chemical compositions of the essential oils were analyzed by GC–MS. The compounds which were 1% or greater in each oil are presented in Table 5. The minor compounds which were which were less than 1% are not presented. The most widely distributed compound was

β-Caryophyllene, a sesquiterpenes, which was found in all the oils except that extracted from *O. gratissimum T. nobilis* and *Z. chalybeum*.

4.2.3 Growth Inhibitory effects of the essential oils

The essential oils from the ten plants were assessed for their growth inhibitory effects on Gram-negative periodontopathic bacteria *A. actinomycetemcomitans, P. gingivalis* and Gram-positive cariogenic bacteria *S. mutans, L. acidophilus* using broth dilution assay. *B. megaterium* (Gram-positive) was included in the tests as a reference strain to represent a non-oral pathogenic bacterium. The growth inhibitory effects of the oils are presented in Figure 6. Most of the tested organisms were sensitive to many of the oils and the chlorhexidine positive control, apart from *L. acidophilus*, whose growth was not significantly inhibited by any of the oils (Figure 6, A and B). However, the growth of *L. acidophilus* was inhibited by the chlorhexidine positive control at the three tested concentrations of 1%, 0.2% and 0.05% with p values of < 0.0001, 0.0001, 0.0107 respectively (Figure 6 B). The most sensitive organism was *A. actinomycetemcomitans*, as its growth was markedly inhibited by six of the plant oils and the chlorhexidine positive controls at all the three concentration tested with $p < 0.0001$ (Figure 6 A). This was followed by *P. gingivalis,* which was markedly inhibited by five of the oils and the chlorhexidine control at the three concentrations tested with $p < 0.0001$(Figure 6 A).

Essential oil from the C. *nardus* plant exhibited the highest activity with complete growth inhibition of the oral pathogens *A. actinomycetemcomitans*, *P. gingivalis* and the non-oral pathogenic *B. megaterium* at all the three concentrations tested (Figure 6, A and B). It also showed inhibition to the growth of *S. mutans* at all the three concentrations with $p < 0.0001$ (Figure 6 B).

Table 5. Chemical composition of the essential oil obtained from the aromatic medicinal plants

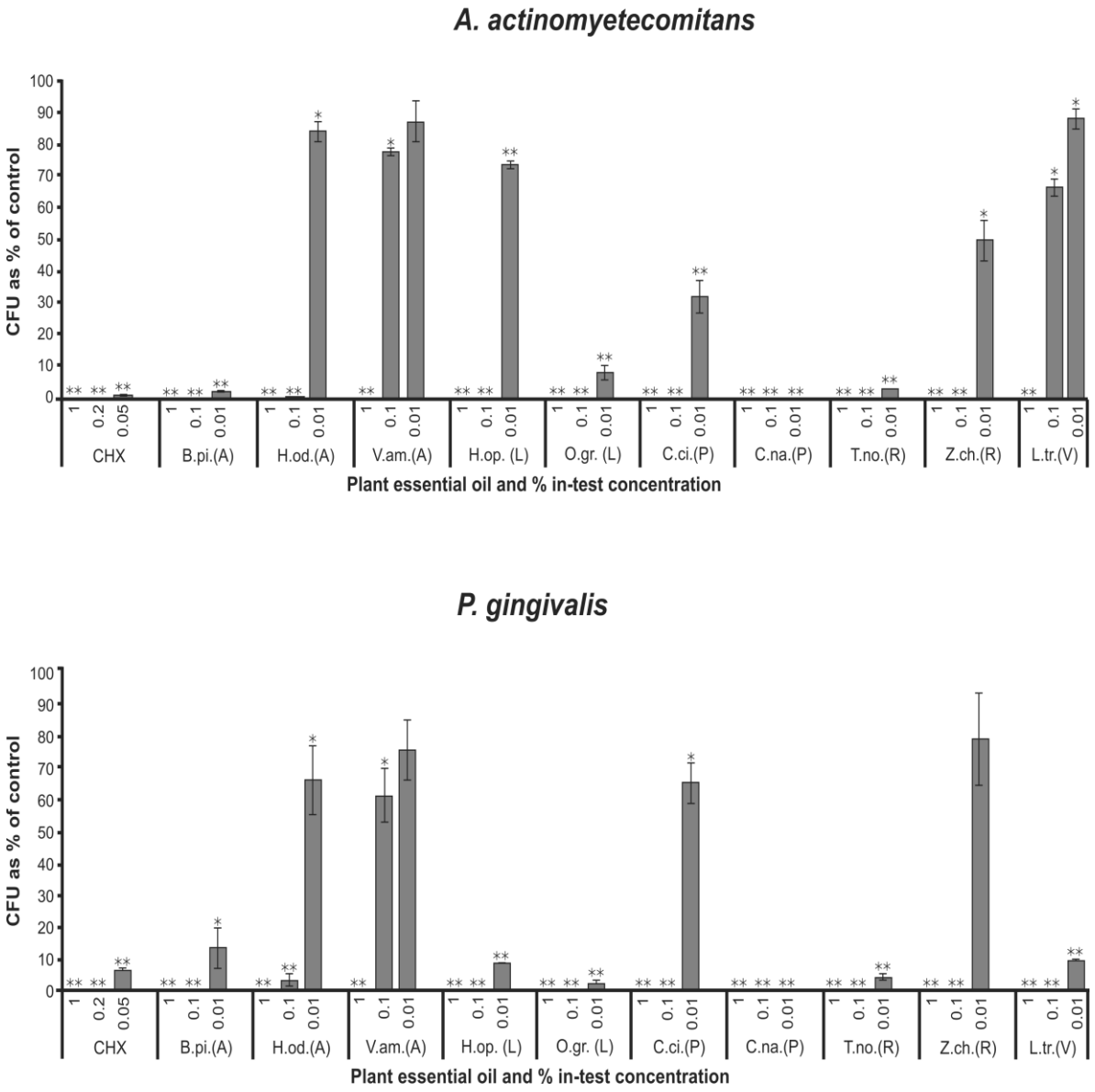
	Percentage of constituents in essential oil[a]									
Name of constituent	[A][b] B.pi[c]	[A] H.od	(A) V.am	[L] H.op	[L] O.gr	[P] C.ci	[P] C.na	[(R] T.no	[R] Z.ch	[V] L.tr
Monoterpenes										
3-Carene									8.3	
4-Carene									2.8	
Limonene									2.5	
Myrcene						10.2	10.5			
Cis-β-Ocimene					3.7				2.1	
Trans-β-Ocimene			3.8		7.6			8.5		
α-Phellandrene									5.1	
β-Phellandrene									1.5	
α-Pinene		4.2							1.1	
β-Pinene									2.6	3.0
Terpinolene									1.4	
Oxygenated Monoterpenes										
Artemiseole						1.6				
1,8-Cineol									1.1	
Citronellal									1.1	
Geranial						35.7			13.3	
Geranic acid						7.2				
Geraniol						3.8			2.4	
Geranyl acetate									1.5	
Linalool	1.4				1.0	1.3			6.4	
Neral						28.6			9.9	
Nerolic acid						2.5				
Terpinene-4-ol									22.3	
α-Terpineol									2.2	
Cis-β- Terpineol,			4.0						1.2	2.4
Sesquiterpenes										
α-Bulnesene		2.8								
Cadinene	3.8				1.7					
δ-Cadinene		7.0		11.4				7.3		
Calarene							4.6			
β-Caryophyllene	12.6	12.6	5.9	10	3.5		1.0			8.4
Cedrene	1.5		3.0							3.3
α-Copaene		7.3		4.6	2.0					
β-cubebene	11.7				10.9					
γ-Elemene							1.6	2.4		1.5
β-Elemene							1.9			
E,E -α-Farnesene			3.6							4.9
β-Farnesene					5.5					1.2
Germacrene D			27.5	28.7			1.3	54.4		23.7
α-Gurjunene								4.9		
Humulene		14.1		24.4						2.9
α-Muurolene							4.3			
β-Patchoulene							4			
Selina-3,7(11)-diene		3.3								
Thujopsene	4.6									
Oxygenated Sesquiterpenes										
α-Cadinol	1.3			1.8			2.1	9.1		1.2
tau.-Cadinol								2.0		
Elemol							1.8			
Eudesmol							1.8			
Germacrene D-4-ol							8.6			
(-)-Globulol	1.0									
Guaiol							1.2			
Intermedeol							43.7			
Ledol					1.2					
Levomenol		7.3								
Nerolidol	1.1		2.4					1.9		
3-Methyl-4-(1,3,3-trimethyl-7-oxa-bicyclo[4.1.0]hept-2-yl)-but-3-en-2-one							3.4			
Muurolol								3.4		
Diterpene										
Phytol		1.6	2.3	4.3				1.2		5.9
Aromatic compounds										
Elixene	5.1									
Eugenol			18.3		56.4					3.7
Methyl isoeugenol								1.7		
Aromatic compound	24.4									

Table 2 continued

	Percentage of constituents in essential oil									
Name of constituent	[A] B.pi	[A] H.od	(A) V.am	[L] H.op	[L] O.gr	[P] C.ci	[P] C.na	[(R] T.no	[R] Z.ch	[V] L.tr
Aliphatic Compounds										
Decanal									1.1	
19,19-Dimethyl-eicosa-8,11-dienoic acid		3.8								
3,4-Dimethyl-1-hexene			2.4							1.1
Ethyl linolenate		3.9		3.7						18.4
Methyl linolenate										3.1
1-Hexanol	2.2									
2-Hexen-1-ol	1.2									
2-Hexenal	1.6									
6-Methyl-3-heptanol										2.7
6-Methyl-5-hepten-2-one						1.5				
Methyl octadec-9-en-12-ynoate		2.2								
Myristic acid		1.4								
3-Octanol			3.9							
1-Octen-3-ol			13.9		1.1					
Palmitic acid		27.1	5.9	10.2				2.1		11.2
2-Undecanone						1.1				
Others[d]	26.5	1.4	3.1	1.0	5.4	6.5	8.2	1.1	10.1	1.4

[a] Expressed as percentage of the peak area relative to the total peak area and only constituents which were 1% or larger are shown.
[b] Plant family names: [A] = Asteraceae; [L] =Lamiaceace; [P] = Poaceae; [R] =Rutaceae; [V] =Verbencaeace.
[c] Plant species names: B.pi = *Bidens pilosa*; H.od= *Helichrysum odoratissimum;* V.am= *Vernonia amygdalina;* H.op= *Hoslundia opposita;* O.gr= *Ocimum gratissimum;* C.ci= *Cymbopogon citratus;* C.na= *Cymbopogon nardus;* T.no= *Teclea nobilis*. Delile; Z.ch= *Zanthoxylum chalybeum;* L.tr= *Lantana trifolia*
[d] Other compounds which were less than 1% in the oil.

A


A. actinomyetecomitans
CFU as % of control
100
90
80
70
60
50
40
30
20
10
0
1
0.2
0.05
CHX
0.1
0.01
B.pi.(A)
H.od.(A)
V.am.(A)
H.op. (L)
O.gr. (L)
C.ci.(P)
C.na.(P)
T.no.(R)
Z.ch.(R)
L.tr.(V)
Plant essential oil and % in-test concentration
P. gingivalis
CFU as % of control
Plant essential oil and % in-test concentration

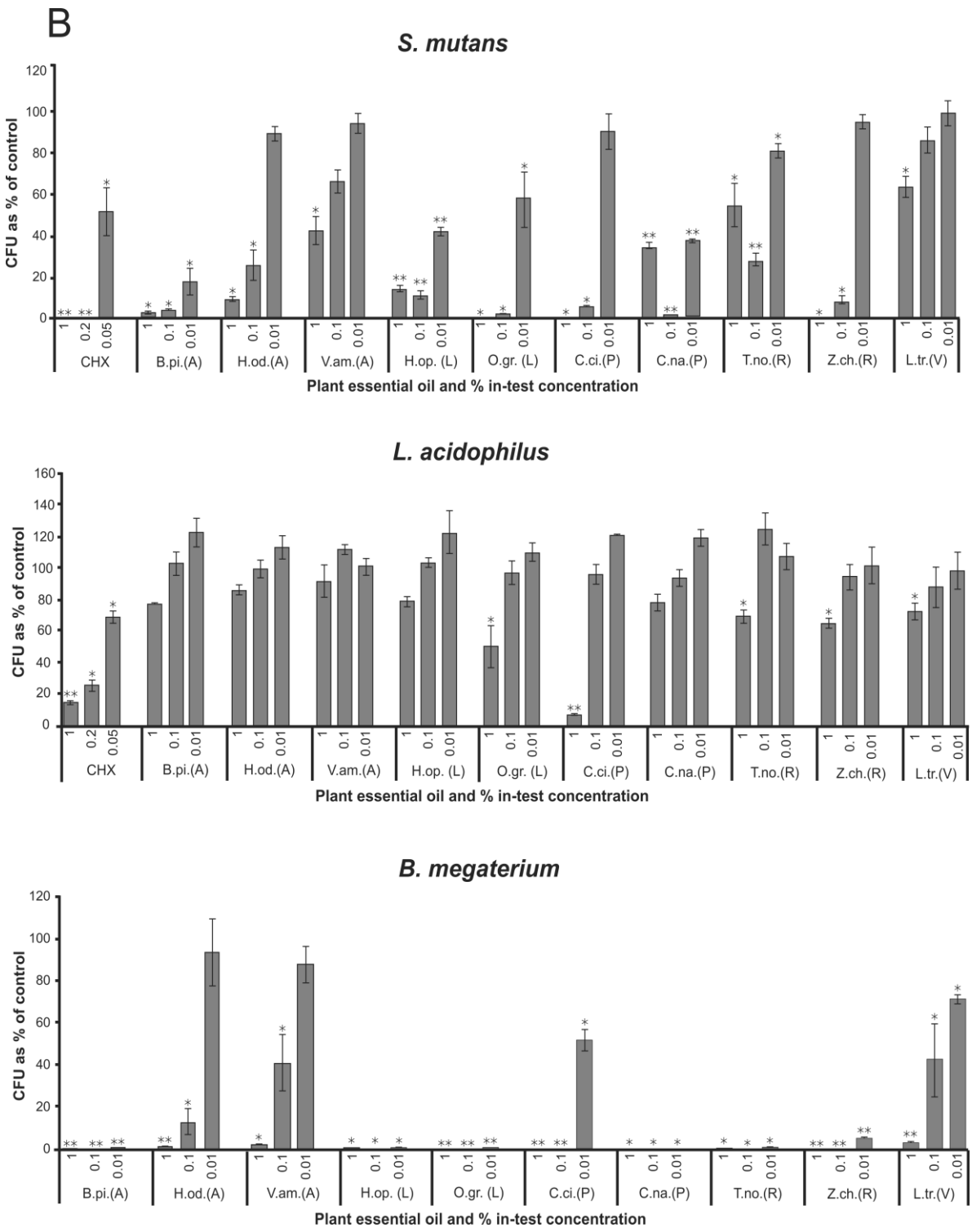


Figure 6. Inhibitory effects of plant essential oils on bacterial growth determined by colony forming units (CFU) assay. The individual bars show the number of surviving bacteria expressed as a percentage of control (n= 4, error bar = S.E.M). Differences in CFU in the control plate and CFU in each tested concentration of the oil or chlorhexidine (in the original dataset) statistically analyzed using the Independent Student's t-test: * = $p < 0.05$ to 0.0001, ** = $p < 0.0001$ compared with the control. The plant essential oil in-test concentration is in percentage of final assay volume. (A) Gram-negative bacteria: *A. actinomycetemcomitans, P. gingivalis.* (B) Gram-positive bacteria: *S. mutans,* L. *acidophilus, B. megaterium.* Positive control: CHX= Chlorhexidine. Plant species names: B.pi = *Bidens pilosa;* H.od= *Helichrysum odoratissimum;* V.am= *Vernonia amygdalina;* H.op= *Hoslundia opposita;* O.gr= *Ocimum gratissimum;* C.ci= *Cymbopogon citratus;* C.na= *Cymbopogon nardus;* T.no= *Teclea nobilis*. Delile; Z.ch= *Zanthoxylum chalybeum;* L.tr= *Lantana trifolia.* Plant family names: (A) = Asteraceae; (L) =Lamiaceace; (P) = Poaceae; (R) =Rutaceae; (V) =Verbencaeace

4.3 STUDY III

4.3.1 Cytotoxicity of the essential oils on human gingival fibroblast

We analyzed the cytotoxicity (IC_{50} values) of the essential oils from *B. pilosa, C. nardus Z. chalybeum* and *O. gratissimum* on human gingival fibroblasts presented in Table 6. The IC_{50} values differed significantly between the four oils (P=0.0003). Tukeys post hoc comparisons test indicated statistically significant differences in mean IC_{50} values of all the oil except that of *B. pilosa* and *O. gratissimum*. The essential oil from *C. nardus* essential oil was the least cytotoxic. The overall rating of cytotoxicity was thus *Z. chalybeum* >O. gratissimum ≥ *B. pilosa* > *C. nardus*

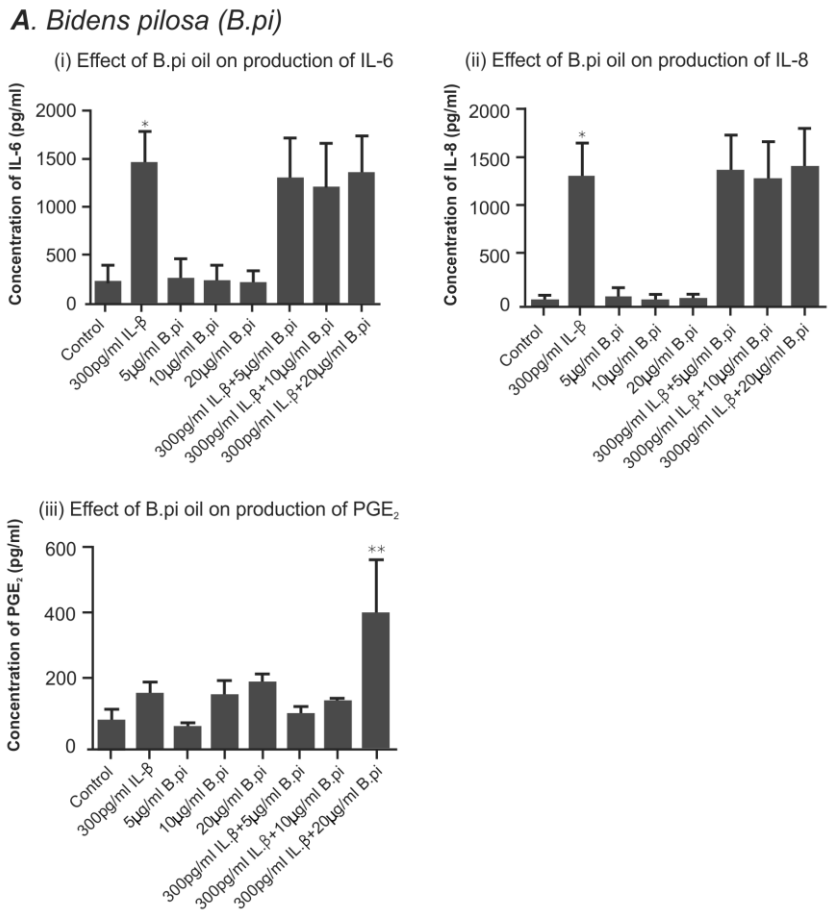
Table 6. IC_{50} values of the essential oils on human gingival fibroblasts

Plant essential oil	**Cytotoxicity (IC_{50} values in mg/ml)**
Bidens pilosa	0.038 ± 0.007
Cymbopogon nardus	0.050 ± 0.004
Ocimum gratissimum	0.036 ± 0.002
Zanthoxylum chalybeum	0.026 ± 0.003

4.3.2 Effect of essential oils on IL-1β-induced secretion of IL-6, IL-8 and PGE_2 in human gingival fibroblasts.

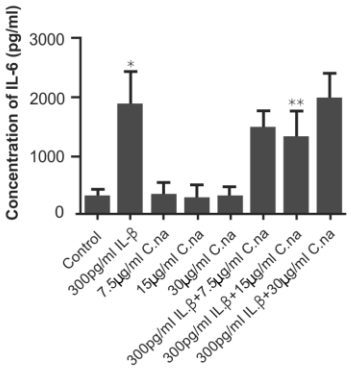
Using human gingival fibroblast cells exposed to IL-1β to provoke an inflammatory response, we assessed the effects of essential oils at sub-cytotoxicity concentrations on cytokines and chemokine secretion and the results are presented in Figure 7. In the absence of IL-1β (control), there was baseline secretion of IL-6, IL-8 and PGE_2. Cells exposed to IL-1β (300pg/ml) showed, as expected, increased secretion of IL-6, IL-8 and PGE_2 compared to unexposed cells (Figure 7). Essential oil from *B. pilosa* had no effect on both baseline and IL-1β induced secretion of IL-6 and IL-8 (Figure 7 A (i) and (ii)). However, the oil increased the secretion of PGE_2 at baseline, but compared with control the increase was not statistically significant (Figure 7 A (iii)). Essential oils from *B. pilosa* had synergistic effects with IL-1β on the

secretion of PGE_2 at concentrations of 20µg/ml ($P < 0.05$) (Figure 7 A (iii)). Essential oil from C. *nardus* had no effect on baseline secretion of IL-6, IL-8 and PGE_2 (Figure 7 B). However, this oil exhibited statistically significant decrease in IL-β induced IL-6 secretion specifically at 15µg/ml concentrations compared to cells exposed to IL-β alone (Figure 7 B (i)). The oil also exhibited a tendency to decrease IL-β induced IL-8 secretion with increasing concentrations (7.5, 15, 30µg) by 20%, 21% and 39% respectively, but the decreases were not statistically significant (Figure 7 B (ii)). Essential oil from *C. nardus*, had synergistic effects with IL-1β on the secretion of PGE_2 specifically at 30µg/ml concentrations ($P < 0.05$) (Figure 7 B (iii)). Essential oil from *Z. chalybeum* had no effect on baseline secretion of IL-6, IL-8 and PGE_2 (Figure 7 C). *Z. chalybeum* oil, however, exhibited statistically significant decreased in IL-β induced IL-6 secretion specifically at 5µg/ml concentration ($P < 0.05$) (Figure 7 C (i)). Essential oil from *O. gratissimum* had no effect on baseline secretion of IL-6 and IL-8 (Figure 7 D (i) and (ii)). The oil, however, decreased baseline secretion of PGE_2 with increasing concentrations (5, 10, 20 µg/ml) by 23%, 32% and 43% respectively, with statistically significant decreases at 10µg/ml and 20µg/ml concentrations compared with the control (Figure 7 D (iii)). The oil also significantly decreased IL-β induced PGE_2 secretion by 59-63% ($P < 0.05$), but there was no specific dose- response relationship (Figure 7 D (iii)).

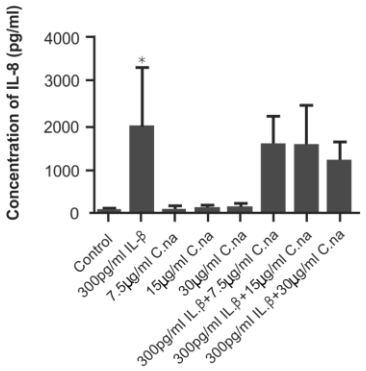


B. *Cymbopogon nardus (C.na)*

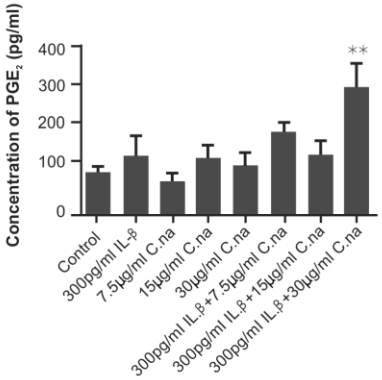
(i) Effect of C.na oil on production of IL-6



(i) Effect of C.na oil on production of IL-8

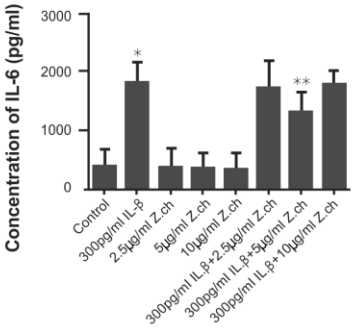


(iii) Effect of C.na oil on production of PGE_2

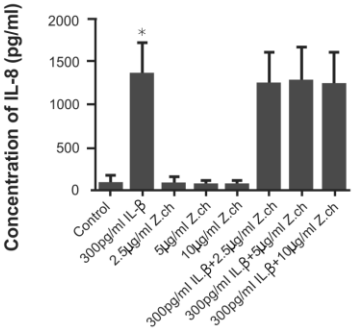


C*. Zanthoxylum chalybeum (Z.ch)*

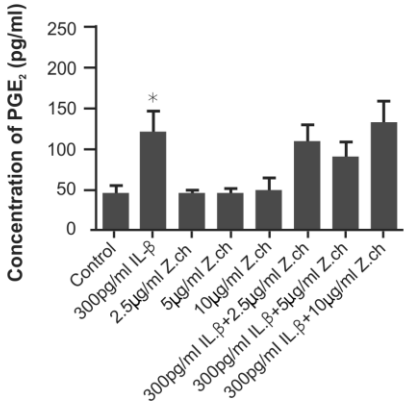
(i) Effect of Z.ch oil on production of IL-6



(ii) Effect of Z.ch oil on production of IL-8



(iii) Effect of Z.ch oil on production of PGE_2



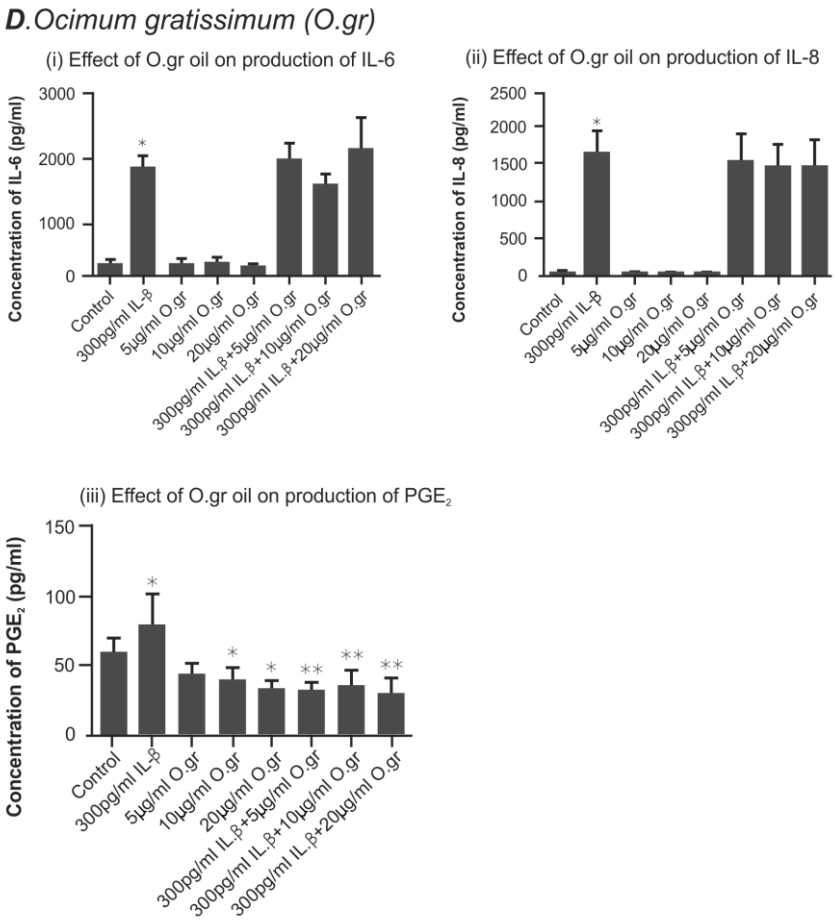


Figure 7. Effect of essential oils from Ugandan medicinal plants on IL-1β-induced secretion of IL-6, IL-8 and PGE_2 in human gingival fibroblasts. Human gingival fibroblasts were treated with IL-1β alone (300pg/ml), essential oils at increasing sub-cytotoxicity concentrations, or combination of IL-1β (300pg/ml) and essential oils. Untreated cells were used as control. IL-6, IL-8, PGE_2 secretions was assessed by enzyme-linked immunosorbent (ELISA). The data are the means ± standard deviations of triplicate assays for three independent experiments. Statistical significance was determined using one-way ANOVA and tukey's post hoc test. *$P < 0.05$ compared with the untreated control, **$P < 0.05$ compared with cells stimulated with 300 pg/ml IL-β alone.

5 DISCUSSION

5.1 DISCUSSION OF MAIN FINDINGS

The major findings in this thesis were that fresh pulp juice from *Z. chalybeum* and *E. latidens* showed activity against all the bacterial species tested, and at all strengths (Table 2), thus confirming that chewing sticks prepared from fresh roots of these plants produce some anti-bacterial effects on oral bacteria. To the best of our knowledge, this is the first time the anti-bacterial effects of extracts from *E. latidens* is being reported. However, there are also reports that fresh root samples from a closely related species, *Euclea natalensis*, have anti-bacterial effects on *S. mutans* and deep periodontal pocket isolates (Stander and Van Wyk, 1991). The *Eucela* genus is known to contain naphthoquinones (Joubert et al., 2006), which are phenolic compounds with significant anti-bacterial properties (Babula et al., 2007). It is therefore probable that the activity exhibited by *E. latidens* in this study is due to the presence of naphthoquinones.

This is also the first time anti-bacterial effects is shown on fresh root samples from *Z. chalybeum* (synonyms*: Fagara chalybea* (Engl.) Engl, *Fagara olitoria* (Engl.) Engl, *Fagara fischeri* Engl.). Most studies on anti-bacterial effects of Z. *chalybeum* (Matu and van Staden, 2003; Olila et al., 2001) or closely related species (Wolinsky and Sote, 1983) have used solvent extracts prepared from dried plant materials or different bacterial species, thus making comparisons with this finding difficult. The genus *Zanthoxylum* are deciduous shrubs and trees, comprising of more than 200 species that are widely distributed, mainly in the temperate and tropical regions (Negi et al., 2011). Many of the species in this genus are rich in alkaloids, especially the benzophenanthridine alkaloids, which are compounds with diverse biological activities, including anti-microbial (Ming Ng et al., 1987; Patiño et al., 2012). The activity of the *Z. chalybeum* species observed in this study could probably also be attributed to alkaloids.

The fact that fresh pulp juice from *Z. chalybeum* and *E. latidens* were active at 50% and 25% strength is significant. The roots from these two plants are used as chewing sticks for brushing teeth and during normal use, the extracts or juices produced are subjected to the diluting action of saliva. So, the 50 % and 25% strengths of fresh pulp juice tested was an attempt to approximately mimic the conditions in the mouth after a reasonable period of exposure. It can therefore be argued that, in addition to mechanical effects, there are also associated anti-bacterial activities which should reduce the cariogenic and periodontopathic bacterial load in

the mouth. However, clinical studies are still warranted to determine whether the extracts kill bacteria *in vivo.*

We had rationalized that demonstration of anti-bacterial effects of fresh extracts from some plants against bacteria associated with dental caries and periodontal diseases would promote the use of these plants as cheap alternative to western toothbrush/toothpaste. In this study, anti-bacterial effects of fresh *Z. chalybeum* and *E. latidens* were demonstrated in the roots. As common in most communities, medicinal plants are usually not cultivated, but harvested from the wild. (Zschocke et al., 2000). Harvesting of plant roots, if not controlled can easily led to extinction of plant species. It is therefore absolutely important that any future promotion of the use of these plants for oral health care should be accompanied by sustainable harvesting strategies.

We also demonstrated anti-bacterial effects on some solvent extracts prepared from dry plant materials. The important finding was that hexane extract from the aerial part of *H. odoratissimum* exhibited good anti-bacterial effects on all the bacterial species tested (MIC 0.0125 to 0.5 mg/ml) except *A. actinomycetemcomitans* (Table 3). This the first report on anti-bacterial effects of hexane extract from this plant species. Previous studies had work on the methanol extract and isolated 3-O-Methylquercetin as an active principle with anti-microbial activity (Van Puyvelde et al., 1989). The low MIC demonstrated by the hexane extract on several bacterial species is a clear indication that the extract has very potent active principles which could be of importance in the treatment or prevention of dental infections caused by these bacterial species.

We found that methanol extract from the leaves *L. trifolia* was active on all bacterial species tested with MIC ranging from 0.25 to 1 mg/ml (Table 3). Previously studies also reported anti-microbial activity from this extract and the compound Umuhengerin was isolated as the active principles (Rwangabo et al., 1988). Umuhengerin was found to have activity on several microorganisms including *Staphylococcus aureus*, *Salmonella typhimurium, Candida tropicalis, Aspergillus niger, Aspergillus fumigatus* and *Trichophyton mentagrophytes* (Rwangabo et al., 1988). The concentration of active principles in the leaves is particularly important for sustainability of the species (Zschocke et al., 2000).

Other solvent extracts which showed activity on at least 3 or more bacterial species were methanol extract from aerial part of *H. odoratissimum,* methanol extract from aerial part of

C. nardus, methanol and hexane extracts from root bark of *Z. chalybeum* and hexane extract from the leaves of *L. trifolia*. Generally, we found more activities in the methanol extracts that in the water or hexane extracts. This is probably because methanol is a solvent with amphiphilic characteristics and can therefore dissolve both polar and non-polar molecules. The larger the variety of compounds that are extracted by the solvent, the better the chance to extract biologically active components (Eloff, 1998)

In this thesis, essential oils from the aromatics medicinal plants were also investigated for their inhibitory effects on the cariogenic *S. mutans, L. acidophilus* and periodontopathic *P. gingivalis,* and *A. actinomycetemcomitans*. As a first step towards assessing their potential use, we measured the essential oil yields from the plants at the time of extraction. Ascertaining essential oil yield is significant because not all plants produce enough essential oil to justify future research efforts and/or commercialization. In our study, the lowest oil yield was obtained from *B. pilosa* (0.05%) and the highest from the two *Cymbopogon* species, namely *C. nardus* (0.36% w/w) and *C. citratus* (0.39 % w/w). Other reports have confirmed the *Cymbopogon* species to produce high amount of essential oils, though the yields are usually affected by external factors like geographical location, climate, nature of the soil, age of the plant, time of collection and mode of extraction oil (Akhila, 2009).

Besides the high yield, C. *nardus* essential oil also exhibited the highest activity with complete growth inhibition of periodontopathic bacteria *P. gingivalis* and *A. actinomycetemcomitans* at all the three concentrations tested (Figure 6 A). It also showed inhibition to the growth of *S. mutans* at all the three concentrations with $p < 0.0001$ (Figure 6 B). Previous reports described essential oil from C. *nardus* to be bactericidal to the human pathogens *Escherichia coli* strain NCTC 10418, and *Staphylococcus aureus,* though at a concentration of 0.25 % (v/v) (Hammer et al., 1999). GC-MS analysis revealed the major constituents in *C. nardus* oil to be dominated by oxygenated sesquiterpenes, specifically intermedeol (Table 5). In a separate bioassay-guided fractionation experiment using a non-oral bacteria *Bacillus subtilis,* we confirmed the compound intermedeol as one of the bioactive molecules in this essential oil (Toom, 2014). However, the activity of intermedeol on oral bacteria is yet to be established.

Other essential oils which were considered promising were those which markedly inhibited growth of at least two bacteria at all the three concentrations tested with $p < 0.0001$ and these oils were from the following plants species: *T. nobilis, H. opposita, O. gratissimum,* and *B.*

pilosa. Generally, the essential oils in this study showed marked growth inhibitory effects on the Gram-negative periodontopathic bacteria *A. actinomycetemcomitans* and *P. gingivalis* than the Gram- positive cariogenic bacteria *S. mutans* and *L. acidophilus*. This findings is in line with another study that found essential oil from *Salvodra persica* to have high killing activity against Gram-negative periodontopathic bacteria *A. actinomycetemcomitans* and *P. gingivalis* and less activity on Gram- positive cariogenic bacteria *S. mutans* and *L. acidophilus* (Sofrata et al., 2011). However, the finding is contrary to reports that Gram-negative bacteria are less susceptible to anti-bacterial agents due to the presence of an outer membrane surrounding their cell walls which restrict diffusion of hydrophobic compounds through lipopolysaccharide covering (Nazzaro et al., 2013). The sensitivity of Gram-negative bacteria to the essential oils has been suggested to be due to the presence of some individual components in the oil which has both lipophilic as well as electrophilic properties and this enable them to penetrate through the outer bacterial membrane (Sofrata et al., 2011).

The marked growth inhibitory effects of most of these essential oils on Gram-negative periodontopathic bacteria *A. actinomycetemcomitans* and *P. gingivalis* suggest the oils could be worth exploring for possible application in treatment of periodontal diseases. The tissue degrading inflammation that destroy periodontal tissues in individuals susceptible to periodontitis is known to be initiated by these specific Gram-negative bacteria (Yucel-Lindberg and Bage, 2013). It has thus been suggested that a treatment approach that can directly control the bacterial infection and at the same time modulate the inflammatory response would be a better option in the management of periodontitis (Tonetti and Chapple, 2011). On this basis, we investigated the anti-inflammatory potentials of essential oils that had shown promising anti-bacterial effects on the Gram-negative periodontopathic bacteria. The essential oils were investigated for their ability to reduce production of pro-inflammatory cytokines (IL-6, IL-8 and prostaglandin E_2) by human gingival fibroblasts. An important finding was that essential oil from O. *gratissimum* significantly decreased induced and baseline secretion of PGE_2 (Figure 7 D (iii)), suggesting possible application of this oil in treatment of periodontitis.

The synthesis of PGE_2 is regulated via 3 groups of enzymes, namely, phospholipase A_2, cyclooxygenase (COX), and prostaglandin E synthase (PGES). The phospholipase A_2 enzymes catalyze the conversion of membrane lipids to arachidonic acid, which is further converted to prostaglandin H_2 (PGH_2) by the two COX isoforms (COX-1 and COX-2). COX-1 is responsible for the baseline levels of PGE_2 while COX-2 produces induced PGE_2. The

terminal step from PGH_2 to PGE_2 is catalyzed by PGES enzymes (Noguchi and Ishikawa, 2007). Our findings that *O. gratissimum* essential oil decreased both baseline and induced PGE_2 secretions suggest that the oil acts upstream by inhibition of either the phospholipase A_2 or the COX enzymes function. We had shown in Study II that *O. gratissimum* essential oil contain several compounds, the major ones being eugenol (56.4%), and β-Cubebene (10.9%). The compound eugenol had been shown to inhibit arachidonic acid metabolism in platelets via the cyclooxygenase pathway (Saeed et al., 1995). It is therefore probable that the observed effect of *O. gratissimum* essential oil on both the basal and induced PGE_2 secretions could partly be due to the major compound eugenol found in this oil. The analgesic property of eugenol has also been attributed to its ability to inhibit PGE_2 synthesis (Thompson and Eling, 1989). In Uganda, fresh leaves from *O. gratissimum* is traditionally used in the treatment of toothache (Table 1). Our findings that the oil extracted from the leaves of *O. gratissimum* was able to reduce basal and induced PGE_2 in gingival fibroblast cells may lend credence to the traditional use of the plant in treatment of toothache.

Besides their effects on the production of pro-inflammatory cytokines, the cytotoxicity of the four promising essential oils on the human gingival fibroblasts was also important consideration in this thesis. Using the IC_{50} values (Table 6), we rated the cytotoxicity of the essential oils from the plants as *Z. chalybeum* > *O. gratissimum* ≥ *B. pilosa* > *C. nardus*. The oil from *C. nardus* was the least cytotoxic (IC_{50} =0.050 mg/ml) because more of this oil was required to kill a given number of cells compared to other oils. In study II, we established the compound Intermedeol as a major component in the *C. nardus* essential oil. It is not clear whether the cytotoxicity of the *C. nardus* essential oil is due to this major component, however, studies have looked at the cytotoxicity of intermedeol on different cell lines and found the IC_{50} values to range from 0.012 mg/ml to 0.077 mg/ml. Future studies of the individual isolated major components found in *C. nardus* oil should include the effects of these components on human gingival fibroblast in order to get more insights into their toxicities.

Essential oils are complex mixtures of different molecules and each oil may contain between 20 and 70 components which are usually of low molecular weights and at different concentrations. Most molecules are present in traces, while two to three are often the most representative components, accounting for 20–70% of the whole oils. The components, including the traces, have their specific roles in the oil. Some components may be present to neutralize the toxic effects of other components. It is therefore not surprising that some oil

components when isolated become more toxic (Bakkali et al., 2008). Some of the oil components have been suggested to be responsible for causing allergic reactions in some individuals (Aberer, 2008; Ventura et al., 2006). There are reports that these allergic reactions are mostly due to the sesquiterpnoid fractions in the oil which occur as a result oxidation of aged or improperly stored oil (Hammer et al., 2006; Mitchell et al., 1972). However, the reactions are known to occur to a very limited fractions of the human populations and can be minimized by avoiding formation of oxidation products in the oil through proper storage (Hammer et al., 2006).

In summary, the major findings as outlined in this thesis give insights into the efficacy and safety of extracts from the Ugandan medicinal plants in the treatment of periodontal diseases and dental caries. With the tremendous increase in interest in the use of natural plant-based remedies worldwide, the findings should encourage more research work geared towards value-addition and commercialization of the promising plants extracts. Some of these endeavors may include isolation and characterization of the active principles in the extracts, more toxicological studies and development of products for clinical trials. The potential socio-economic contribution of these medicinal plants, in long-term, could be enormous. This is especially because some of the plants which have exhibited promising anti-bacterial and/or anti-inflammatory effects like *H. odoratissimum*, *C. nardus* and *O. gratissimum* are road-side plants and are currently regarded as weeds in the plantation gardens, with little or no value attached to them. With value-addition and commercialization, these plants could turn into cash crops, thus creating opportunities for employment and wealth creation.

5.2 METHODOLOGICAL CONSIDERATIONS

5.2.1 Study 1 and II

In study I and II, all appropriate measures were taken to ensure that the aims for which the studies were conducted were achieved. During literature search, only plants that had both their local and scientific names mentioned in literature were picked for the study. During collection, all possible efforts were made to ensure that the plants were collected from exact districts in Uganda where they are being used, as mentioned in the literature. If there was need for additional plant materials during the experiments, all efforts were again made to collect the particular plant from the exact area where it was first collected. Voucher specimens were archived for future follow-up.

We also made efforts to test the plant extracts using the recommended methods, and these were the agar dilution assay (for solvent extracts) and broth dilution assay (for essential oils) (Rios et al., 1988). There is no recommended method for testing anti-bacterial activity in fresh pulp juice, so we utilized the agar well-diffusion assay. The agar well-diffusion assay was used, in this case, because of its simplicity and capacity to analyze samples quickly and cheaply. However, the method has limitations in that it can only detect activity of substances that can easily diffuse into the agar media (Barry, 1986). Thus, it is possible that active lipophilic substances in the fresh pulp juice that could not diffuse into the agar media were not detected. This could have impacted on the number of active extracts from the fresh plants observed in this study. Future methodological studies to determine appropriate method for anti-bacterial activity testing in fresh pulp juice could be considered.

In both study I and II, the anti-bacterial effects of the plant extracts were investigated on planktonic bacteria. However, the periodontopathic and cariogenic bacteria are clinically present in the oral cavity as a biofilm. Recent understandings of biofilm characteristics reveal that bacteria living in the biofilm are naturally tolerant to anti-bacterial agents than the planktonic counterparts (Sbordone and Bortolaia, 2003). The anti-bacterial effects of the plant extracts on the planktonic bacteria observed in this study may therefore not exactly reflect their effects in *vivo*. Future studies on the effects of these extracts on the bacteria in their natural ecosystem could be considered.

5.2.2 Study III

In selecting plants for study III, considerations were given to ease of availability of the species and the plant family. For instance, in Study II, essential oil from the plant *T. nobilis* (Family: Rutaceae family) had shown marked growth inhibitory effects on the Gram-negative bacteria and should have been therefore considered for further analysis in Study III. However, this plant species seem to be getting extinct and it is extremely difficult to get. In this respect, we replaced the plant T. *nobilis* with *Z. chalybeum*, another member of the Rutaceae family, which had also shown good growth inhibitory effects on the Gram-negative bacteria, and is readily available. Similarly, *H. opposita*, and *O. gratissimum* are all from the Lamiaceace family and all of them had demonstrated marked growth inhibitory effects on the Gram-negative bacteria. However, *O. gratissimum* is readily available, thus it was picked for further analysis in study III.

In study III, in *vitro* cytotoxicity testing of the essential oils were done on the host cells as recommended by the International Organization for Standardization (ISO, 2009) and other authors (Cos et al., 2006). The cells in the oral cavity that may come into direct contact with the plant essential oils during use are the barrier epithelial cells and the extracellular matrix/collagen synthesizing fibroblast cells. Both the epithelial and the fibroblast cells also acts as accessory immunes cells and therefore do participate in the local inflammations via their productions of cytokines (Takashiba et al., 2003). Both cells type were thus appropriate for anti-inflammatory and cytotoxicity testing. In this study, we chose to use primary human gingival fibroblasts, grown as monolayer cultures in cell culture flasks. Gingival fibroblast monolayer cultures is a useful model that substituted for the real live tissues of the oral cavity in our situation. However, cells in the culture system are restricted in growth to a two-dimensional carrier and are not surrounded by a morphologic organized extracellular matrix/collagen and other interacting cells types as seen in real live tissues (Hillmann et al., 1999). As a result, these cells tend to be more sensitive to drugs or other stimuli than cells in *vivo*. The effects of chlorhexidine digluconate on human gingival fibroblasts is a classic example. Chlorhexidine is a widely used mouth-rinse solutions. However, studies have shown that the concentrations of chlorhexidine which are highly cytotoxic in *vitro* generally do not harm cells in *vivo* (Pucher and Daniel, 1993). It is therefore possible that the cytotoxic effects of the essential oil extracts on human fibroblasts observed in this study may not truly reflect the in *vivo* situations, given the fact that some of these plants have been used traditionally for hundreds of years without documented adverse effects.

6 CONCLUSION

- Besides the mechanical effects, there is also anti-bacterial effects that is assocaited with brushing teeth with chewing stickss prepared from fresh roots of *Z. chalybeum* and *E. latidens*. However, further clinical studies are warranted to determine the effects of chewing sticks prepared from these two plants in *vivo*.

- The hexane extract from the aerial part of *H. odoratissimum* has very potent active principles that could be of importance in the treatment or prevention of dental caries and periodontal diseases. Other solvent extracts that demonstrated good activty and thus warranting further investigations are: methanol extract from aerial part of *H. odoratissimum*; methanol extract from aerial part of *C. nardus;* methanol and hexane extracts from root bark of *Z. chalybeum*; hexane extract from the leaves of *L. trifolia*.

- The essential oils from the following plants showed marked growht inhibitoy effects on at least two bacterial species and are thus considered promising: *C. nardus, T. nobilis, H. opposita, O. gratissimum,* and *B. pilosa*. Their marked growth inhibitory on esspecially on Gram-negative periodontopathic *A. actinomycetemcomitans* and *P. gingivalis* suggest the oils could be worth exploring for possible application in treatment of periodontal diseases.

- The essential oils from the test plants showed cytotoxicity to human gingival fibroblasts cells, with oil from *Z. chalybeum* being the most cytotoxic and oil from *C. nardus* the least cytotoxic. The overall rating of cytotoxicity of the essential oil from the tested plants was thus *Z. chalybeum* > *O. gratissimum* ≥ *B. pilosa* > *C. nardus*.

- Essential oil extracted *O. gratissimum* demonstrated both anti-bacterial and anti-inflamatory effects suggesting the oil worth exploring for possible application in treatmnt of periodontitis.

7 FUTURE PERSPECTIVES

Clinical studies are warranted to determine the in *vivo* anti-bacterial effects of fresh extracts from roots of *Z. chalybeum* and *E. latidens.*

The strong anti-bacterial effects of the hexane extract from the aerial part of *H. odoratissimum* against cariogenic and periodontopathic bacteria calls for further studies to isolate and identify the active principles in this extract. Other extracts needing further studies to isolate and identify their active principles are: methanol extract from aerial part of *C. nardus;* Hexane extract from the leaves of *L. trifolia*.

Further anti-bacterial and cytotoxicity studies on the individual major components of the promising essential oils to explore if the components can be used alone or in combination with other components. Further studies should also explore combination of different essential oils to see if there is considerable enhancement of anti-bacterial effects compared to individual oils

Further studies on the cytotoxic effects of the promising essential oils on keratinocytes.

The possibility of a natural products with has both anti-bacterial and anti-inflammatory effects is promising. Further studies should look into developing the oil from *O. gratissimum* into some products and testing the anti-bacterial and anti-inflammatory effects in *vivo*.

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